DESCRIPTION

MANIPULATION OF GENES OF THE MEVALONATE AND ISOPRENOID PATHWAYS TO CREATE NOVEL TRAITS IN TRANSGENIC ORGANISMS

Cross-Reference to a Related Application

This application claims the benefit of U.S. Provisional Application No. 60/221,703, filed July 31, 2000.

Field of the Invention

This invention relates to the fields of biotechnology and genetic engineering, in particular to agricultural and aquacultural biotechnology. More specifically, the invention relates to transgenic plants and microalgae, in particular to transplastomic plants and microalgae and means for insertion of genetic material into plastids.

Background of the Invention

The ubiquitous isoprenoid biosynthetic pathway is responsible for the formation of the most chemically diverse family of metabolites found in nature (Hahn *et al.*, J. Bacteriol. 178:619-624, 1996) including sterols (Popjak, Biochemical symposium no. 29 (T. W. Goodwin, ed.) Academic Press, New York, pp17-37, 1970), carotenoids (Goodwin, Biochem. J. 123:293-329, 1971), dolichols (Matsuoka *et al.*, J. Biol. Chem. 266:3464-3468, 1991), ubiquinones (Ashby and Edwards, J. Biol. Chem. 265:13157-13164, 1990), and prenylated proteins (Clarke, Annu. Rev. Biochem. 61:355-386, 1992). Biosynthesis of isopentenyl diphosphate (IPP), the essential 5-carbon isoprenoid precursor, occurs by two distinct compartmentalized routes in plants (Lange and Croteau, Proc. Natl. Acad. Sci. USA 96:13714-13719, 1999). In the plant cytoplasm, IPP is assembled from three molecules of acetyl coenzyme A by the well-characterized mevalonate pathway (Lange and Croteau, Proc. Natl. Acad. Sci. USA 96:13714-13719, 1999). However, a recently discovered mevalonate-independent pathway is responsible for the synthesis of IPP in plant chloroplasts (Lichtenthaler *et al.* FEBS Letters 400:271-274, 1997).

Following the synthesis of IPP via the mevalonate route, the carbon-carbon double

bond must be isomerized to create the potent electrophile dimethylally diphosphate (DMAPP). This essential activation step, carried out by IPP isomerase, insures the existence of the two 5-carbon isomers, IPP and DMAPP, which must join together in the first of a series of head to tail condensation reactions to create the essential allylic diphosphates of the isoprenoid pathway (Hahn and Poulter, J. Biol. Chem. 270:11298-11303, 1995). Recently, it was reported that IPP isomerase activity was not essential in *E. coli*, one of many eubacteria containing only the non-mevalonate pathway for the synthesis of both 5-carbon isomers, suggesting the existence of two separate mevalonate-independent routes to IPP and DMAPP (Hahn *et al.*, J. Bacteriol. 181:4499-4504, 1999). Thus, it is unclear whether an IPP isomerase is essential for the synthesis of isoprenoids in plant plastids as well. Regardless of whether IPP isomerase activity is present in plant plastids, the separation by compartmentalization of the two different biosynthetic routes, the mevalonate and deoxyxylulose phosphate pathways (or "non-mevalonate"), for IPP and DMAPP biosynthesis in plants is the fundamental tenet upon which the subject inventions are based.

The synthesis of IPP by the mevalonate pathway (Eisenreich *et al.*, Chemistry and Biology 5:R221-R233, 1998) is cytoplasm based and occurs as follows: The condensation of two acetyl CoA molecules to yield acetoacetyl CoA is catalyzed by acetoacetyl CoA thiolase (EC 2.3.1.9). The addition of another molecule of acetyl CoA to acetoacetyl CoA is catalyzed by 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) synthase (EC 4.1.3.5) to yield HMG-CoA, which is reduced in the subsequent step to mevalonate by HMG-CoA reductase (EC 1.1.1.34). Mevalonate is phosphorylated by mevalonate kinase (EC 2.7.1.36) to yield phosphomevalonate, which is phosphorylated, by phosphomevalonate kinase (EC 2.7.4.2) to form mevalonate diphosphate. The conversion of mevalonate diphosphate to IPP with the concomitant release of CO2 is catalyzed by mevalonate diphosphate decarboxylase (EC 4.1.1.33).

In organisms utilizing the deoxyxylulose phosphate pathway (aka "non-mevalonate pathway", "methylerythritol phosphate (MEP) pathway", and "Rohmer pathway"), the five carbon atoms in the basic isoprenoid unit are derived from pyruvate and D-glyceraldehyde phosphate (GAP) (Eisenreich *et al.*, 1998). Thus, synthesis of IPP and/or DMAPP by the non-mevalonate route, which occurs in plastids, is as follows: Pyruvate and GAP are condensed to give 1-deoxy-D-xylulose 5-phosphate (DXP) by

DXP synthase (Sprenger *et al.*, Proc. Natl. Acad. Sci. USA 94:12857-12862, 1997). The rearrangement and reduction of DXP to form 2-C-methylerythritol 4-phosphate (MEP), the first committed intermediate in the non-mevalonate pathway for biosynthesis of isoprenoids is catalyzed by DXP reductoisomerase (Kuzuyama *et al.*, Tetrahedron Lett. 39:4509-4512, 1998). MEP is then appended to CTP to form 4-(cytidine 5'-diphospho)-2- C-methyl-D-erythritol (Rohdich *et al.*, Proc. Natl. Acad. Sci. USA 96:11758-11763, 1999), followed by phosphorylation of the C2 hydroxyl group (Lüttgen *et al.*, Proc. Natl. Acad. Sci. USA 97:1062-1067, 2000) and elimination of CMP, to form a 2,4-cyclic diphosphate (Herz *et al.*, Proc. Natl. Acad. Sci. USA 97:2486-2490, 2000). Interestingly, Herz *et al.* reported the possible existence of bifunctional proteins with both YgbP and YgbB activities. Once the remaining steps to the fundamental five-carbon isoprenoid building blocks, IPP and DMAPP, in the non-mevalonate pathway are discovered, they will serve as additional targets for inhibitors with antiobiotic and herbicidal activity.

Since the non-mevalonate pathway is ultimately responsible for the biosynthesis of compounds critical for photosynthesis such as the prenyl side-chain of chlorophylls, which serve as lipophillic anchors for the photoreceptors and the photoprotective carotenoid pigments, any enzyme, gene, or regulatory sequence involved in the biosynthesis of IPP and/or DMAPP can be a potential target for herbicides. For example, the antibiotic fosmidomycin, a specific inhibitor of the enzyme DXP reductoisomerase (Kuzuyama *et al.*, Tetrahedron Lett. 39:7913-7916, 1998) has been shown to have significant herbicidal activity, especially in combination with other herbicides (Kamuro *et al.* "Herbicide" U.S. Patent No. 4,846,872; issued July 11, 1989). The report of an Arabidopsis thaliana albino mutant being characterized as a disruption of the CLA1 gene, later revealed as encoding DXP synthase by Rohmer *et al.* (Lois *et al.*, Proc. Natl. Acad. Sci. USA 95:2105-2110, 1998), also illustrates the potential of non-mevalonate pathway enzymes as targets for compounds with herbicidal activity. Accordingly, one of ordinary skill in the art can readily understand that as additional compounds are discovered exhibiting herbicidal activity based on their effects on the non-mevalonate pathway, those compounds could be used in accord with the teachings herein.

The synthesis of carotenoids from IPP and DMAPP takes place in plant plastids by a genetically- and enzymatically-defined pathway (Cunningham and Gantt, Ann. Rev.

Plant Mol. Biol. 39:475-502, 1998). Enhanced production of carotenoids such as lycopene and ß-carotene in plants is highly desirable due to the reported health benefits of their consumption (Kajiwara *et al.*, Biochem. J. 324:421-426, 1997). Enhanced carotenoid production in plants can also have a dramatic effect on their coloration and be highly desirable to the growers of ornamentals, for example. The IPP isomerase reaction is considered to be a rate-limiting step for isoprenoid biosynthesis (Ramos-Valdivia *et al.*, Nat. Prod. Rep. 6:591-603, 1997). Kajiwara *et al.* reported that the expression of heterologous IPP isomerase genes in a strain of *E. coli* specifically engineered to produce carotenoids resulted in over a 2-fold increase in ß-carotene formation. Recently, it has been reported that expression of an additional gene for DXP synthase in an *E. coli* strain specifically engineered to produce carotenoids also increased the level of lycopene substantially (Harker and Bramley, FEBS Letters 448:115-119, 1999). Increased isoprenoid production also has been shown in bacteria by combining carotenogenic genes from bacteria with an orf encoding IPP isomerase; and was even further enhanced when additionally combined with the dxs gene from the MEP pathway to supply the precursors IPP and DMAPP (Albrecht *et al.* Nature Biotechnology 18: 843- 846, 2000).

Accumulation of one specific isoprenoid, such as beta-carotene (yellow-orange) or astaxanthin (red-orange), can serve to enhance flower color or nutriceutical composition depending if the host is cultivated as an ornamental or as an output crop; and if the product accumulates in the tissue of interest (*i.e.* flower parts or harvestable tissue). In plants, tissue with intrinsic carotenoid enzymes can accumulate ketocarotenoids such as astaxanthin in chromoplasts of reproductive tissues of tobacco by addition of the biosynthetic enzyme beta-carotene ketolase (Mann *et al.*, Nature Biotechnology 18: 888-892, 2000). Astaxanthin is the main carotenoid pigment found in aquatic animals; in microalgae it accumulates in the Chlorophyta such as in species of Haematococcus and Chlamydomonas. Thus, an increase in the essential 5- carbon precursors, IPP and DMAPP, by expression of orfs encoding IPP isomerase and orfs upstream thereof, can feed into the production output of such valuable isoprenoids in organisms other than bacteria.

As a further example of utility, Petunia flower color is usually due to the presence of modified cyanidin and delphinidin anthocyanin pigments to produce shades in red to blue groupings. Recently produced yellow seed-propagated multiflora and grandiflora

petunias obtain their coloration from the presence of beta-carotene, lutein and zeaxanthin carotenoid pigments in combination with colorless flavonols (Nielsen and Bloor, Scienia Hort. 71: 257-266, 1997). Industry still lacks bright yellow and orange clonally propagated trailing petunias. Metabolic engineering of the carotenoid pathway is desired to introduce these colors in this popular potted and bedding plant.

Plant genetic engineering has evolved since the 1980s from arbitrarily located monocistronic insertions into a nuclear chromosome, often subject to multiple copies, rearrangements and methylation, to predetermined sites for defined multicistronic or multigenic operon insertions into a plastid chromosome (plastome), which thus far is thought impervious to typical nuclear gene inactivation. While breeding of crop plants by nuclear genome engineering is nevertheless a proven technology for major agronomic crops and for traits such as herbicide resistance, introgression of genes into the plastome is a highly promising breeding approach for several reasons as described by Bock and Hagemann (Bock and Hagemann, Prog. Bot. 61:76-90, 2000). Of note is the containment of transgenes in the transplastomic plant: Plastids are inherited through the maternal parent in most plant species and thus plastid-encoded transgenes are unable to spread in pollen to non-target species. Therefore plastid engineering can minimize negative impacts of genetically engineered plants. A report on potential transfer by pollen of herbicide resistance into weedy relatives of cultivated crops (Keeler *et al.*, Herbicide Resistant Crops: Agricultural, Economic, Environmental, Regulatory and Technological Aspects, pp. 303-330, 1996) underscores the value of using plastid engineering rather than nuclear engineering for critical production traits such as herbicide resistance. Daniell *et al.* have recently demonstrated herbicide resistance through genetic engineering of the chloroplast genome (Daniell *et al.*, Nat. Biotechnol., 16:345-348, 1998).

Moreover, plastids are the site of essential biosynthetic activity. Although most associate photosynthesis as the primary function of the chloroplast, studies document that the chloroplast is the center of activity for functions involving carbon metabolism, nitrogen metabolism, sulfur metabolism, biochemical regulation, and various essential biosynthetic pathways including amino acid, vitamin, and phytohormone biosynthesis. Crop traits of interest such as nutritional enhancement require genetic manipulations that impact plastid biosynthetic pathways such as carotenoid production. While nuclear-encoded gene products can be exported from the engineered nucleus into the

plastid for such manipulations, the biosynthetic genes themselves can be inserted into the plastid for expression and activity. As we begin to pyramid multiple genes often required for pathway manipulations (such as the aforementioned carotenoid biosynthesis) the repeated use of selection markers is expected to lead to unstable crops through homology-dependent gene silencing (Meyer and Saedler, Ann. Rev. Plant. Physiol. Mol. Biol. 47:23-48, 1996). In addition, the requirement for higher expression levels of transgenes for effective phenotypes such as vitamin levels and herbicide and pest resistance levels often falls short in nuclear transformations. These deficiencies are overcome through plastid transformation or combining plastid with nuclear transformations: The plastid recognizes strings of genes linked together in multicistronic operons and, due to the high copy number of genes within a plastid and within plastids in a cell, can produce a hundred- to thousand-fold the amount of transgene product. Accordingly, there is a continuing need for improved methods of producing plants having transformed plastids (transplastomic plants).

Golden rice is one example for which plastid engineering can complement nuclear engineering of pathways that reside in the plastid, yet have met with limited success. The metabolic pathway for beta-carotene (pro-vitamin A) was assembled in rice plastids by introduction into the nuclear genome of four separate genes, three encoding plastid-targeted proteins using three distinct promoters, plus a fourth selectable marker gene using a repeated promoter (Ye *et al.* Science 287:303-305, 2000). The wild-type rice endosperm is free of carotenoids but it does produce geranylgeranyl diphosphate; combining phytoene synthase, phytoene desaturase, and lycopene-beta cyclase resulted in accumulation of beta-carotene to make "golden rice". However, the quantity produced was lower than the minimum desired for addressing vitamin A deficiency. An increased supply of precursors for increasing intermediates, such as geranylgeranyl diphosphate, is predicted to significantly increase isoprenoid production. Insertion of an operon encoding the entire mevalonate pathway into the rice plastome of the "golden rice" genotype, using for example the methods as described in Khan and Maliga, Nature Biotechnology 17: 910-914, 1999, can provide a means for making improvements in metabolic engineering of this important monocot crop.

Proplastid and chloroplast genetic engineering have been shown to varying degrees of homoplasmy for several major agronomic crops including potato, rice, maize, soybean,

grape, sweet potato, and tobacco including starting from non-green tissues. Non-lethal selection on antibiotics is used to proliferate cells containing plastids with antibiotic resistance genes. Plastid transformation methods use two plastid-DNA flanking sequences that recombine with plastid sequences to insert chimeric DNA into the spacer regions between functional genes of the plastome, as is established in the field (see Bock and Hagemann, Prog. Bot. 61:76-90, 2000, and Guda *et al.*, Plant Cell Reports 19:257-262, 2000, and references therein).

Antibiotics such as spectinomycin, streptomycin, and kanamycin can shut down gene expression in chloroplasts by ribosome inactivation. These antibiotics bleach leaves and form white callus when tissue is put onto regeneration medium in their presence. The bacterial genes *aad*A and *neo* encode the enzymes aminoglycoside-3′-adenyltransferase and neomycin phosphotransferase, which inactivate these antibiotics, and can be used for positive selection of plastids engineered to express these genes. Polynucleotides of interest can be linked to the selectable genes and thus can be enriched by selection during the sorting out of engineered and non-engineered plastids. Consequently, cells with plastids engineered to contain genes for these enzymes (and linkages thereto) can overcome the effects of inhibitors in the plant cell culture medium and can proliferate, while cells lacking engineered plastids cannot proliferate. Similarly, plastids engineered with polynucleotides encoding enzymes from the mevalonate pathway to produce IPP from acetyl CoA in the presence of inhibitors of the non-mevalonate pathway can overcome otherwise inhibitory culture conditions. By utilizing the polynucleotides disclosed herein in accord with this invention, an inhibitor targeting the non-mevalonate pathway and its components can be used for selection purposes of transplastomic plants produced through currently available methods, or any future methods which become known for production of transplastomic plants, to contain and express said polynucleotides and any linked coding sequences of interest.

This selection process of the subject invention is unique in that it is the first selectable trait that acts by pathway complementation to overcome inhibitors. This is distinguished from the state of the art of selection by other antibiotics to which resistance is conferred by inactivation of the antibiotic itself, *e.g.* compound inactivation as for the aminoglyoside 3'-adenyltransferase gene or neo gene. This method avoids the occurrence of resistant escapes due to random insertion of the resistance gene into the nuclear

genome or by spontaneous mutation of the ribosomal target of the antibiotic, as is known to occur in the state of the art. Moreover, this method requires the presence of an entire functioning mevalonate pathway in plastids. For example, if one of the enzyme activities of the mevalonate pathway is not present in the plastid, resistance will not be conferred.

There is strong evidence indicating that the origin of plastids within the cell occurred via endosymbiosis and that plastids are derived from cyanobacteria. As such, the genetic organization of the plastid is prokaryotic in nature (as opposed to the eukaryotic nuclear genome of the plant cell). The plastid chromosome ranges from roughly 110 to 150 Kb in size (196 for the green alga Chlamydomonas), much smaller than that of most cyanobacteria. However, many of the bacterium genes have either been lost because their function was no longer necessary for survival, or were transferred to the chromosomes of the nuclear genome. Most, but not all, of the genes remaining on the plastid chromosome function in either carbon metabolism or plastid genetics. However, many genes involved in these functions, as well as the many other functions and pathways intrinsic to plastid function, are also nuclear encoded, and the translated products are transported from the cytoplasm to the plastid. Studies have documented nuclear encoded genes with known activity in the plastid that are genetically more similar to homologous genes in bacteria rather than genes of the same organism with the same function but activity in the cytoplasm as reviewed for example in Martin *et al.* (1998) Nature 393:162-165 and references therein.

The process whereby genes are transported from the plastid to the nucleus has been addressed. Evidence indicates that copies of many plastid genes are found among nuclear chromosomes. For some of these, promoter regions and transit peptides (small stretches of DNA encoding peptides that direct polypeptides to the plastid) become associated with the gene that allows it to be transcribed, and the translated polypeptide relocated back into the plastid. Once this genetic apparatus has become established, the genes present in the plastid chromosome may begin to degrade until they are no longer functional, *i.e.*, any such gene becomes a pseudogene.

As is common in prokaryotic systems, many genes that have a common function are organized into an operon. An operon is a cluster of contiguous genes transcribed from one promoter to give rise to a polycistron mRNA. Proteins from each gene in the polycistron are then translated. There are 18 operons in the plastid chromosome of

tobacco (Nicotiana tabacum). Although many of these involve as few as two genes, some are large and include many genes. Evolutionary studies indicate that gene loss- as pseudogenes or completely missing sequences- occurs as individuals rather than as blocks of genes or transcriptional units. Thus other genes surrounding a pseudogene in a polycistronic operon remain functional.

The rpl23 operon consists of genes whose products are involved in protein translation. Most of these genes are ribosomal proteins functioning in either the large or small ribosomal subunit. One particular gene of note, infA, encodes an initiation factor protein that is important in initiating protein translation. Although this gene is functional in many plants, it is a pseudogene in tobacco and all other members of that family (Solanaceae), including the horticulturally valuable tomato, petunia, and potato crops. A recent survey of plant groups has indicated that there have been numerous loses of functionality of infA (Millen *et al.*, Plant Cell 13: 645-658, 2001). This as well as other pseudogenes are identified in species whose chloroplast genomes have not yet been fully sequenced.

Pseudogenes such as infA become potential target sequences for insertion of intact orfs. Inserted orfs are controlled by regulatory upstream and downstream elements of the polycistron and are promoterless themselves. Pseudogenes are known for a multiplicity of crops and algae with chloroplast genomes that are already fully sequenced. Crops include grains such as rice and trees such as Pinus. Of note in the latter are the eleven ndh genes; all may serve as potential targets for transgene insertion.

Transplastomic solanaceous crops are highly desirable in order to eliminate the potential for gene transfer from engineered lines to wild species, as demonstrated in Lycopersicon (Dale, P.J. 1992. Spread of engineered genes to wild relatives. Plant Physiol. 100:13-15.). A method for plastid engineering that enables altered pigmentation, for improved nutrition in tomato or improved flower color in Petunia and ornamental tobacco as examples, is desirable for solanaceous crops. The infA gene is widely lost among rosids and some asterids; among the latter, infA is a pseudogene in all solanaceous species examined (representing 16 genera). The solanaceous infA DNA sequences show high similarity, with all nucleotide changes within infA being documented. Thus one set of flanking sequences of reasonable length as known in the art should serve for directed insertion of an individual or multiple orfs into the infA sites of the solanaceous species.

It is documented in a solanaceous species that flanking sequences for genes to be inserted into the plastome are not required to be specific for the target species, as incompletely homologous plastid sequences are integrated at comparable frequencies (Kavanagh *et al.*, Genetics 152:1111-1122, 1999).

The upstream 5' region, often referred to as the 5' UTR, is important on the expression level of a transcript as it is translated. Knowing the translation products of surrounding genes in a polycistron allows one to select a pseudogene site that is affiliated with a strong 5' UTR for optimizing plastid expression in a particular tissue. The plastid genome in many plant species can have multiple pseudogenes that are located in different polycistronic sites. So, if one has a choice, one can select a site based on whether it is actively transcribed in green vs non-green plastid; and then if the polycistron has high or low relative expression in that plastid type. Moreover, monocistronic mRNA of ndhD was detected in developed leaves but not in greening or expanding leaves of barley (Hordeum vulgare), despite this gene being part of a polycistronic unit as reported by del Campo *et al.* (1997) Plant Physiol 114:748. Thus, one can time transgene product production by treating an inactive gene, based on developmental expression, as a pseudogene for targetting and integration purposes using the invention disclosed herein.

Algal species are becoming increasingly exploited as sources of nutraceuticals, pharmaceuticals, and lend themselves to aquaculture. Mass production of the isoprenoid compound astaxanthin produced by the green microalga Haemotcoccus is one successful example of the above. Metabolic engineering that would increase product yields and composition in microalgae would significantly benefit the industry. The development of organellar transformation for the unicellular green alga *Chlamydomonas reinhardtii*, with its single large chloroplast, opens the door for conducting studies on genetic manipulation of the isoprenoid pathway. Filamentous or multicellular algae are also of interest as untapped biofactories, as are other nongreen algae whose pathways for producing unique fatty acids, amino acids, and pigments can be ameliorated for commercial benefit.

The biolistic DNA delivery method is a general means with which to transform the chloroplast of algae (Boynton and Gillham, Methods Enzymol. 217:510-536, 1993). Sequencing of at least six plastomes from algae should facilitate transformation systems by confirming insertion sites, including pseudogene sites, and the regulatory elements directing heterologous gene expression. What is required is a dominant marker for

selection of stable transformants to which natural resistance is absent (Stevens and Purton, J. Phycol 33: 713-722, 1997). For Chlamydomonas, chloroplasts can be engineered using markers that confer spectinomycin resistance following their integration into the plastome via homologous recombination. By utilizing the polynucleotides disclosed herein in accord with this invention, an inhibitor targeting the non-mevalonate pathway and its components can be used for selection purposes of transplastomic algae produced through currently available methods, or any future methods which become known for production of transplastomic algae, to contain and express said polynucleotides and any linked coding sequences of interest. This is a novel selection vehicle for transplastomic algae. Moreover, elevating the supply of essential precursors for isoprenoid production in algae as described above is enabled by this invention.

Summary of the Invention

This invention relates to the presence of enzymatic activities necessary to form IPP from acetyl CoA, generally known as the mevalonate pathway, within plant and microalgae plastids. This invention may also require the presence of IPP isomerase activity within plastids resulting from the insertion into said plants and microalgae of a polynucleotide encoding a polypeptide with IPP isomerase activity. This invention may be achieved by the use of any polynucleotide, be it a DNA molecule or molecules, or any hybrid DNA/RNA molecule or molecules, containing at least one open reading frame that when expressed provides a polypeptide(s) exhibiting said activities within plastids. These open reading frames may be identical to their wild type progenitors, or alternatively may be altered in any manner (for example, with plastid-optimized codon usage), may be isolated from the host organism to be modified, may originate from another organism or organisms, or may be any combination of origin so long as the encoded proteins are able to provide the desired enzymatic activity within the target plastids. The described open reading frames may be inserted directly into plastids using established methodology or any methodology yet to be discovered. Alternatively, plastid localization of the desired activities may be achieved by modifying genes already residing in the cell nucleus, inserting foreign polynucleotides for nuclear residence, or inserting polynucleotides contained on exogenous, autonomous plasmids into the cell cytoplasm so that in all cases their encoded proteins are transported into the plastid. For example, a chloroplast transit

(targeting) peptide can be fused to a protein of interest. Any combination of the above methods for realizing said activities in plant and microalgae plastids can be utilized. By causing the complete mevalonate pathway enzymatic activity to occur in plastids normally possessing only the non-mevalonate pathway, the presence of said activities within the chloroplasts of a specific plant or microalgae will endow it with resistance to a compound, molecule, etc. that targets a component of the non-mevalonate pathway, be it an enzyme, gene, regulatory sequence, etc., thereby also providing a useful selection system based on circumvention of the inhibition of the non-mevalonate pathway in transplastomic plants and microalgae.

In addition, this invention relates to the use of open reading frames encoding polypeptides with enzymatic activities able to convert acetyl CoA to IPP, generally known as the mevalonate pathway, and a polypeptide with IPP isomerase activity as a method for increasing the production of IPP, DMAPP, and isoprenoid pathway derived products whose level within plant and microalgae plastids is dependent on the level of IPP and/or DMAPP present within the plastids. The presence of exogenous genes encoding 1-deoxy-D-xylulose-5-phosphate synthase and IPP isomerase have been shown to increase the production of carotenoids in eubacteria, presumably due to an increased production of IPP and/or DMAPP. Thus, insertion of the entire mevalonate pathway, solely or coupled with an additional IPP isomerase, into plastids will increase the level of IPP and/or DMAPP, resulting in an increased level of carotenoids and other yet to be determined isoprenoid pathway derived products within plant and microalgae plastids. This invention can utilize an open reading frame encoding the enzymatic activity for IPP isomerase independently or in addition to said open reading frames comprising the entire mevalonate pathway to obtain the increased level of isoprenoid pathway derived products within plant and microalgae plastids. This invention may be achieved by the use of any DNA molecule or molecules, or any hybrid DNA/RNA molecule or molecules, containing open reading frames able to provide said activities within plant and microalgae plastids. These open reading frames may be identical to their wild type progenitors, may be altered in any manner, may be isolated from the plant to be modified, may originate from another organism or organisms, or may be any combination of origin so long as the encoded proteins are able to provide said activities within plastids. The described open reading frames may be inserted directly into plant and microalgae plastids using

established methodology or any methodology yet to be discovered. Alternatively, plastid localization of the desired activities may be achieved by modifying genes already residing in the nucleus, inserting foreign genes for nuclear residence, or inserting genes contained on exogenous, autonomous plasmids into the cytoplasm so that in all cases their encoded proteins are transported into the plastid. Any combination of the above methods for realizing said activities in plastids can be utilized.

Further, this invention also relates to the direct insertion of any foreign gene into a plant or microalgae chloroplast by coupling it to the open reading frames encoding polypeptides with enzymatic activities able to convert acetyl CoA to IPP, thus comprising the entire mevalonate pathway. By utilizing a compound, molecule, etc. that targets a component of the non-mevalonate pathway be it an enzyme, gene, regulatory sequence, etc., a method of selection analogous to the use of kanamycin and spectinomycin resistance for the transformation event is achieved. As inhibition of the non-mevalonate pathway in a plant or microalgae results in the impairment of photosynthesis, the presence of the mevalonate pathway biosynthetic capability is apparent, thus enabling the facile screening of concomitant incorporation into plastids of a foreign gene coupled to the open reading frames comprising the entire mevalonate pathway. The use of a polynucleotide comprising an open reading frame encoding a polypeptide with IPP isomerase activity in addition to the open reading frames encoding the mevalonate pathway is a particularly preferred embodiment, which provides all enzymatic activities necessary to synthesize both IPP and DMAPP and overcome the effect(s) of inhibition of the non-mevalonate pathway.

Further, this invention is unique and novel in that the transforming DNA, that is integrated by two or more homologous/heterologous recombination events, is purposefully targeted into inactive gene sites selected based on prior knowledge of transcription in plastid type, developmental expression including post-transcriptional editing, and post-transcriptional stability. Additionally, this invention uses the regulatory elements of known inactive genes (pseudogenes) to drive production of a complete transforming gene unrelated to the inserted gene site. Thus, by utilizing the transgene insertion method disclosed herein in accord with this invention, any foreign gene can be targeted to an inactive gene site (the pseudogene) through currently available methods of gene transfer, or any future methods which become known for production of transgenic

and transplastomic plants, to contain and express said foreign gene and any linked coding sequences of interest. This gene insertion process of the subject invention is unique in that it is the first method specifically acting by pseudogene insertion to overcome the need for promoters and other regulatory elements normally associated with a transforming DNA vector while permitting site-specific recombination in organellar genomes. The use of the infA pseudogene insertion site in the solanaceous crops in particular is a preferred embodiment for the transformation of plastids using the open reading frames for the mevalonate pathway as well as for providing the necessary precursors for modified output traits in plants.

Brief Description of the Drawings

FIG. 1 is a map of cloning vector pFCO1 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), and mevalonate diphosphate decarboxylase (MDD).

FIG. 2 is a map of expression vector pFCO2 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), and mevalonate diphosphate decarboxylase (MDD).

FIG. 3 is a map of cloning vector pHKO1 containing *S. cerevisiae* orf encoding acetoacetyl thiolase (AACT); *A. thaliana* orfs encoding HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGRt).

FIG. 4 is a map of expression vector pHKO2 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), mevalonate diphosphate decarboxylase (MDD), and acetoacetyl thiolase (AACT); *A. thaliana* orfs encoding HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGRt) which in their summation are designated Operon A, encoding the entire mevalonate pathway.

FIG. 5 is a map of cloning vector pHKO3 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), mevalonate diphosphate decarboxylase (MDD), and acetoacetyl thiolase (AACT); *A. thaliana* orfs encoding HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGRt) which in their summation are designated Operon B, encoding the entire mevalonate pathway.

FIG. 6 is an illustration of how the mevalonate (MEV) pathway, by providing an alternative biosynthetic route to IPP, circumvents blocks in the MEP pathway due to a

mutation in the gene for deoxyxylulose phosphate synthase (dxs) and due to inhibtion by fosmidomycin of deoxyxylulose phosphate reductoisomerase (dxr).

FIG. 7 is a map of vector pBSNT27 containing *N. tabcum* chloroplast DNA (cpDNA) and the *N. tabcum infA* pseudogene and pBSNT27 sequence (SEQ ID NO: 17)

FIG. 8 is a map of plastid transformation vector pHKO4 containing *N. tabcum* chloroplast DNA (cpDNA) flanking the insertion of Operon B into the *infA* pseudogene.

FIG. 9 is a map of cloning vector pHKO5 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), and mevalonate diphosphate decarboxylase (MDD), and acetoacetyl thiolase (AACT); *A. thaliana* orfs encoding HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGRt); *R. capsulatus* orf encoding IPP isomerase (IPPI) which in their summation are designated Operon C, encoding the entire mevalonate pathway and IPP isomerase.

FIG. 10 is a map of cloning vector pFHO1 containing *S. cerevisiae* orf encoding acetoacetyl thiolase (AACT); *A. thaliana* orf encoding HMG-CoA synthase (HMGS); *Streptomyces* sp CL190 orf encoding HMG-CoA reductase (HMGR).

FIG. 11 is a map of cloning vector pFHO2 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), and mevalonate diphosphate decarboxylase (MDD), and acetoacetyl thiolase (AACT); *A. thaliana* orf encoding HMG-CoA synthase (HMGS); *Streptomyces sp* CL190 orf encoding HMG-CoA reductase (HMGR) which in their summation are designated Operon D, encoding the entire mevalonate pathway.

FIG. 12 is a map of cloning vector pFHO3 containing *S. cerevisiae* orfs encoding phosphomevalonate kinase (PMK), mevalonate kinase (MVK), and mevalonate diphosphate decarboxylase (MDD), and acetoacetyl thiolase (AACT); *A. thaliana* orf encoding HMG-CoA synthase (HMGS); *Streptomyces sp* CL190 orf encoding HMG-CoA reductase (HMGR); *R. capsulatus* orf encoding IPP isomerase (IPPI) which in their summation are designated Operon E, encoding the entire mevalonate pathway and IPP isomerase.

FIG. 13 is a map of cloning vector pFHO4 containing a *S. cerevisiae* orf encoding acetoacetyl thiolase (AACT) coupled to the *Streptomyces sp* CL190 gene cluster which in their summation are designated Operon F, encoding the entire mevalonate pathway and IPP isomerase.

FIG.14 is is a plastid transformation vector pHKO7 containing *N. tabacum* chloroplast DNA (cpDNA) flanking the insertion of Operon C into the *inf*A pseudogene.

FIG. 15 is a map of expression vector pHKO9 containing Operon B.

FIG. 16 is a map of expression vector pHK10 containing Operon C.

FIG 17 is a map of plastid transformation vector pFHO6 containing *N. tabacum* chloroplast DNA (cpDNA) flanking the insertion of both Operon E and the R. capsulatus orf encoding phytoene synthase (PHS) into the *inf*A pseudogene.

Brief Description of the Sequences

SEQ ID NO: 1) is a PCR primer containing *Saccharomyces cerevisiae* DNA.

SEQ ID NO: 2) is a PCR primer containing S. cerevisiae DNA.

SEQ ID NO: 3) is a PCR primer containing *S. cerevisiae* DNA.

SEQ ID NO: 4) is a PCR primer containing *S. cerevisiae* DNA.

SEQ ID NO: 5) is a PCR primer containing S. cerevisiae DNA.

SEQ ID NO: 6) is a PCR primer containing S. cerevisiae DNA.

SEQ ID NO: 7) is a PCR primer containing Arabidopsis thalian.a DNA.

SEQ ID NO: 8) is a PCR primer containing A. thaliana DNA.

SEQ ID NO: 9) is a PCR primer containing A. thaliana DNA.

SEQ ID NO: 10) is a PCR primer containing *A. thaliana* DNA.

SEQ ID NO: 11) is a PCR primer containing *S. cerevisiae* DNA.

SEQ ID NO: 12) is a PCR primer containing *S. cerevisiae* DNA.

SEQ ID NO: 13) is a Oligonucleotide containing *S. cerevisiae* DNA.

SEQ ID NO: 14) is a Oligonucleotide containing *A. thaliana* and *S. cerevisiae* DNA.

SEQ ID NO: 15) is an Oligonucleotide containing *S. cerevisiae* DNA.

SEQ ID NO: 16) is an Oligonucleotide containing *S. cerevisiae* DNA.

SEQ ID NO: 17) is Vector pBSNT27 containing *Nicotiana tabacum* DNA.

SEQ ID NO: 18) is an Oligonucleotide containing *N. tabacum* and *S. cerevisiae* DNA.

SEQ ID NO: 19) is an Oligonucleotide containing *N. tabacum* and *A. thaliana* DNA.

SEQ ID NO: 20) is a PCR primer containing *Rhodobacter capsulatus* DNA.

SEQ ID NO: 21) is a PCR is a primer containing *R. capsulatus* DNA.

SEQ ID NO: 22) is a PCR primer containing Schizosaccharomyces pombe DNA.

SEQ ID NO: 23) is a PCR primer containing S. pombe DNA.

SEQ ID NO: 24) is a PCR primer containing *Streptomyces sp* CL190 DNA.

SEQ ID NO: 25) PCR is a primer containing *Streptomyces sp* CL190 DNA.

SEQ ID NO: 26) is an Oligonucleotide containing *S. cerevisiae DNA.*

SEQ ID NO: 27) is an Oligonucleotide *containing S.* cerevisiae DNA.

SEQ ID NO: 28) is an Oligonucleotide containing *Streptomyces sp* CL190 and *R. capsulatus* DNA.

SEQ ID NO: 29) is an Oligonucleotide containing *R. capsulatus* DNA.

SEQ ID NO: 30) is an Oligonucleotide containing *Streptomyces sp* CL190 and *S. cerevisiae* DNA.

SEQ ID NO: 31) is an Oligonucleotide containing *Streptomyces sp* CL190 DNA.

SEQ ID NO: 32) is an Oligonucleotide containing *N. tabacum* and *S. cerevisiae* DNA.

SEQ ID NO: 33) is an Oligonucleotide containing *N. tabacum* and *R. capsulatus* DNA.

SEQ ID NO: 34) is an Oligonucleotide containing *N. tabacum* and *S. cerevisiae* DNA.

SEQ ID NO: 35) is an Oligonucleotide containing *N. tabacum* and *S. pombe* DNA.

SEQ ID NO: 36) is an Oligonucleotide containing NotI restriction site.

SEQ ID NO: 37) is an Oligonucleotide containing NotI restriction site.

SEQ ID NO: 38) is an Oligonucleotide containing *S. cerevisiae* DNA.

SEQ ID NO: 39) is an Oligonucleotide containing *A. thaliana* DNA.

SEQ ID NO: 40) is an Oligonucleotide containing *S. cerevisiae* DNA.

SEQ ID NO: 41) is an Oligonucleotide containing *R. capsulatus* DNA.

SEQ ID NO: 42) is an Oligonucleotide containing *S. cerevisiae* DNA.

SEQ ID NO: 43) is an Oligonucleotide containing *S. pombe* DNA.

SEQ ID NO: 44) is an Oligonucleotide containing *R. capsulatus* DNA.

SEQ ID NO: 45) is an Oligonucleotide containing *R. capsulatus* DNA.

SEQ ID NO: 46) is an Oligonucleotide containing *S. pombe* DNA.

SEQ ID NO: 47) is an Oligonucleotide containing *S. pombe* DNA.

SEQ ID NO: 48) is *Saccharomyces cerevisiae* orf for phosphomevalonate kinase (ERG8).

SEQ ID NO: 49) *Saccharomyces cerevisiae* orf for mevalonate kinase (ERG12).

SEQ ID NO: 50) *Saccharomyces cerevisiae* orf for mevalonate diphosphate decarboxylase (ERG19).

SEQ ID NO: 51) Saccharomyces cerevisiae orf for acetoacetyl thiolase.

SEQ ID NO: 52) *Arabidopsis thaliana* orf for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase.

SEQ ID NO: 53) *Arabidopsis thaliana* orf for HMG-CoA reductase.

SEQ ID NO: 54) *Schizosaccharomyces pombe* IDI1 (IPP isomerase).

SEQ ID NO: 55) *Rhodobacter* capsulatus idiB (IPP isomerase).

SEQ ID NO: 56) *Streptomyces sp* CL190 orf encoding HMG-CoA reductase.

SEQ ID NO: 57) *Streptomyces sp* CL190 gene cluster containing mevalonate pathway and IPP isomerase orfs.

SEQ ID NO: 58) Operon A containing *A. thaliana* and *S. cerevisiae* DNA

SEQ ID NO: 59) is Operon B containing *A. thaliana* and *S. cerevisiae* DNA.

SEQ ID NO: 60) is Operon C containing *A. thaliana*, *S. cerevisiae*, and *R. capsulatus* DNA.

SEQ ID NO: 61) is Operon D containing *A. thaliana*, *S. cerevisiae*, and *Streptomycs sp* CL190 DNA.

SEQ ID NO: 62) is Operon E containing *A. thaliana*, *S. cerevisiae*, *Streptomycs sp* CL190 DNA, and R. capsulatus DNA.

SEQ ID NO: 63) is Operon F containing containing *S. cerevisiae* and *Streptomycs sp* CL190 DNA.

SEQ ID NO: 64) is Operon G containing *A. thaliana*, *S. cerevisiae* and *S. pombe* DNA.

SEQ ID NO: 65) is PCR primer containing *R. capsulatus* DNA.

SEQ ID NO: 66) is PCR primer containing *R. capsulatus* DNA.

SEQ ID NO: 67) is an Oligonucleotide containing *N. tabacum* and *R. capsulatus* DNA.

SEQ ID NO: 68) is an Oligonucleotide containing *N. tabacum* and *R. capsulatus*

DNA.

SEQ ID NO: 69) is an Oligonucleotide containing *N. tabacum* and *S. cerevisiae* DNA.

SEQ ID NO: 70) is an Oligonucleotide containing *N. tabacum* and *R. capsulatus* DNA.

SEQ ID NO: 71) is *Rhodobacter* capsulatus orf encoding phytoene synthase (crtB).

SEQ ID NO: 72) is plastid transformation vector pHKO4, containing Operon B, containing *A. thaliana* and *S. cerevisiae* DNA.

SEQ ID NO: 73) is plastid transformation vector pHKO7, containing Operon C, containing *A. thaliana*, *S. cerevisiae*, and *R. capsulatus* DNA.

SEQ ID NO: 74) is plastid transformation vector pHKO8, containing Operon G, containing *A. thaliana*, *S. cerevisiae*, and *S. pombe* DNA.

SEQ ID NO: 75) is plastid transformation vector pFHO5 containing R. capsulatus DNA encoding phytoene synthase.

SEQ ID NO: 76) is plastid transformation vector pFHO6, containing Operon E, containing *A. thaliana*, *S. cerevisiae*, *Streptomycs sp* CL190 DNA, and *R.capsulatus* DNA.

Detailed Description

In the description that follows, a number of terms used in genetic engineering are utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

A protein is considered an isolated protein if it is a protein isolated from a host cell in which it is naturally produced. It can be purified or it can simply be free of other proteins and biological materials with which it is associated in nature, for example, if it is recombinantly produced.

An isolated nucleic acid is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally

occurring genomic DNA molecule, but is not flanked by both of the coding or noncoding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic or plastomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic or plastomic DNA; (c) a separate molecule such as a cDNA, a genomic or plastomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in mixtures of (i) DNA molecules, (ii) transfected cells, and (iii) cell clones, e.g., as these occur in a DNA library such as a cDNA or genomic DNA library.

One DNA portion or sequence is downstream of second DNA portion or sequence when it is located 3' of the second sequence. One DNA portion or sequence is upstream of a second DNA portion or sequence when it is located 5' of that sequence.

One DNA molecule or sequence and another are heterologous to one another if the two are not derived from the same ultimate natural source, or are not naturally contiguous to each other. The sequences may be natural sequences, or at least one sequence can be derived from two different species or one sequence can be produced by chemical synthesis provided that the nucleotide sequence of the synthesized portion was not derived from the same organism as the other sequence.

A polynucleotide is said to encode a polypeptide if, in its native state or when manipulated by methods known to those skilled in the art, it can be transcribed and/or translated to produce the polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

A nucleotide sequence is operably linked when it is placed into a functional relationship with another nucleotide sequence. For instance, a promoter is operably linked to a coding sequence if the promoter effects its transcription or expression. Generally, operably linked means that the sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, it is well known that certain genetic elements, such as enhancers, may be operably linked even at a distance, i.e., even if not contiguous.

In a plastome, sequences are physically linked by virtue of the chromosome

configuration, but they are not necessarily operably linked due to differential expression for example. Transgenes can be physically linked prior to transformation, or can become physically linked once they insert into a plastome. Transgenes can become operably linked if they share regulatory sequences upon insertion into a plastome.

The term recombinant polynucleotide refers to a polynucleotide which is made by the combination of two otherwise separated segments of sequence accomplished by the artificial manipulation of isolated segments of polynucleotides by genetic engineering techniques or by chemical synthesis. In so doing one may join together polynucleotide segments of desired functions to generate a desired combination of functions.

The polynucleotides may also be produced by chemical synthesis, e.g., by the phosphoramidite method described by Beaucage and Caruthers (1981) *Tetra. Letts.*, 22:1859-1862 or the triester method according to Matteuci *et al.* (1981) *J. Am. Chem. Soc.*, 103: 3185, and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Polynucleotide constructs prepared for introduction into a prokaryotic or eukaryotic host will typically, but not always, comprise a replication system (i.e. vector) recognized by the host, including the intended polynucleotide fragment encoding the desired polypeptide, and will preferably, but not necessarily, also include transcription and translational initiation regulatory sequences operably linked to the polypeptide-encoding segment. Expression systems (expression vectors) may include, for example, an origin of replication or autonomously replicating sequence (ARS) and expression control sequences, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, and mRNA stabilizing sequences. Signal peptides may also be included where appropriate, preferably from secreted polypeptides of the same or related species, which allow the protein to cross and/or lodge in cell membranes or be secreted from the cell.

Variants or sequences having substantial identity or homology with the polynucleotides encoding enzymes of the mevalonate pathway may be utilized in the

practice of the invention. Such sequences can be referred to as variants or modified sequences. That is, a polynucleotide sequence may be modified yet still retain the ability to encode a polypeptide exhibiting the desired activity. Such variants or modified sequences are thus equivalents. Generally, the variant or modified sequence will comprise at least about 40%-60%, preferably about 60%-80%, more preferably about 80%-90%, and even more preferably about 90%-95% sequence identity with the native sequence.

Sequence relationships between two or more nucleic acids or polynucleotides are generally defined as sequence identity, percentage of sequence identity, and substantial identity. See, for example, "Pedestrian Guide to Analyzing Sequence Data Bases" at www.embl-heidelberg.de/~schneide/paper/springer96/springer.html. In determining sequence identity, a "reference sequence" is used as a basis for sequence comparison. The reference may be a subset or the entirety of a specified sequence. That is, the reference sequence may be a full-length gene sequence or a segment of the gene sequence.

Methods for alignment of sequences for comparison are well known in the art. See, for example, Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; Needleman *et al.* (1970) *J. Mol. Biol.* 48:443; Pearson *et al.* (1988) *Proc. Natl. Acad. Sci.* 85:2444; CLUSTAL in the PC/Gene Program by Intelligenetics, Mountain View, California; GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA. Preferred computer alignment methods also include the BLASTP, BLASTN, and BLASTX algorithms. See, Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410.

"Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. "Percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions as compared to the reference window for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of

positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Polynucleotide sequences having "substantial identity" are those sequences having at least about 50%-60% sequence identity, generally at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described above. Preferably sequence identity is determined using the default parameters determined by the program. Substantial identity of amino acid sequence generally means sequence identity of at least 50%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Nucleotide sequences are generally substantially identical if the two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH. Nucleic acid molecules that do not hybridize to each other under stringent conditions may still be substantially identical if the polypeptides they encode are substantially identical. This may occur, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

As noted, hybridization of sequences may be carried out under stringent conditions. By "stringent conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60° C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary stringent conditions include hybridization with a buffer solution of 30 to 35% formamide, 1.0 M NaC1, 1% SDS (sodium dodecyl sulphate) at 37◦ C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaC1/0.3 M trisodium citrate) at 50 to 55◦ C. It is recognized that the temperature, salt, and wash conditions may be altered to increase or decrease stringency conditions. For

the post-hybridization washes, the critical factors are the ionic strength and temperature of the final wash solution. See, Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284.

As indicated, fragments and variants of the nucleotide sequences of the invention are encompassed herein. By "fragment" is intended a portion of the nucleotide sequence. Fragments of the polynucleotide sequence will generally encode polypeptides which retain the biological/enzymatic activity of the native protein. Those of skill in the art routinely generate fragments of polynucleotides of interest through use of commercially available restriction enzymes; synthetic construction of desired polynucleotides based on known sequences; or use of "erase-a-base" technologies such as *Bal* 31 exonuclease, by which the skilled artisan can generate hundreds of fragments of a known polynucleotide sequence from along the entire length of the molecule by time-controlled, limited digestion. Fragments that retain at least one biological or enzymatic activity of the native protein are equivalents of the native protein for that activity.

By "variants" is intended substantially similar sequences. For example, for nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of an enzyme of the mevalonate pathway. Variant nucleotide sequences include synthetically derived sequences, such as those generated for example, using site-directed mutagenesis. Generally, nucleotide sequence variants of the invention will have at least 40%, 50%, 60%, 70%, generally 80%, preferably 85%, 90%, up to 95% sequence identity to its respective native nucleotide sequence. Activity of polypeptides encoded by fragments or variants of polynucleotides can be confirmed by assays disclosed herein.

"Variant" in the context of proteins is intended to mean a protein derived from the native protein by deletion or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or human manipulation. Conservative amino acid substitutions will generally result in variants that retain biological function. Such variants are equivalents of the native protein. Variant proteins that retain a desired biological activity are encompassed within the subject invention. Variant proteins of the invention may include those that are altered in various ways including amino acid substitutions, deletions,

truncations, and insertions. Methods for such manipulation are generally known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods and Enzymol*;. 154:367-382; and the references cited therein.

An expression cassette may contain at least one polynucleotide of interest to be cotransformed into the organism. Such an expression cassette is preferably provided with a plurality of restriction sites for insertion of the sequences of the invention to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The cassette may include 5' and 3' regulatory sequences operably linked to a polynucleotide of interest. By "operably linked" is intended, for example, a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. When a polynucleotide comprises a plurality of coding regions that are operably linked such that they are under the control of a single promoter, the polynucleotide may be referred to as an "operon".

The expression cassette will optionally include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a polynucleotide sequence of interest and a transcriptional and translational termination region functional in plants or microalgae. The transcriptional initiation region, the promoter, is optional, but may be native or analogous, or foreign or heterologous, to the intended host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native organism into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcriptional initiation region that is heterologous to the coding sequence.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot

(1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) Gene 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

Where appropriate, the polynucleotides of interest may be optimized for expression in the transformed organism. That is, the genes can be synthesized using plant or algae plastid-preferred codons corresponding to the plastids of the plant or algae of interest. Methods are available in the art for synthesizing such codon optimized polynucleotides. See, for example, U. S. Patent Nos. 5,380,831 and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference. Of course, the skilled artisan will appreciate that for the transplastomic purposes described herein, sequence optimization should be conducted with plastid codon usage frequency in mind, rather than the plant or algae genome codon usage exemplified in these references.

It is now well known in the art that when synthesizing a polynucleotide of interest for improved expression in a host cell it is desirable to design the gene such that its frequency of codon usage approaches the frequency of codon usage of the host cell. It is also well known that plastome codon usage may vary from that of the host plant or microalgae genome. For purposes of the subject invention, "frequency of preferred codon usage" refers to the preference exhibited by a specific host cell plastid in usage of nucleotide codons to specify a given amino acid. To determine the frequency of usage of a particular codon in a gene, the number of occurrences of that codon in the gene is divided by the total number of occurrences of all codons specifying the same amino acid in the gene. Similarly, the frequency of preferred codon usage exhibited by a plastid can be calculated by averaging frequency of preferred codon usage in a number of genes expressed by the plastid. It usually is preferable that this analysis be limited to genes that are among those more highly expressed by the plastid. Alternatively, the polynucleotide of interest may be synthesized to have a greater number of the host plastid's most preferred codon for each amino acid, or to reduce the number of codons that are rarely used by the host.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation.

Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region), Elroy-Stein *et al.* (1989) *PNAS USA* 86:6126-6130; potyvirus leaders, for example, TEV leader (Tobacco Etch Virus), Allison *et al.* (1986); MDMV Leader (Maize Dwarf Mosaic Virus) *Virology* 154:9-20; and human immunoglobulin heavy-chain binding protein (BiP), Macejak *et al.* (1991) *Nature* 353:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), Jobling *et al.* (1987) *Nature* 325:622-625; tobacco mosaic virus leader (TMV), Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256; and maize chlorotic mottle virus leader (MCMV), Lommel *et al.* (1991) *Virology* 81:382-385. See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing an expression cassette, the various polynucleotide fragments may be manipulated, so as to provide for the polynucleotide sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the polynucleotide fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous nucleotides, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

In addition, expressed gene products may be localized to specific organelles in the target cell by ligating DNA or RNA coded for peptide leader sequences to the polynucleotide of interest. Such leader sequences can be obtained from several genes of either plant or other sources. These genes encode cytoplasmically-synthesized proteins directed to, for example, mitochondria (the F1-ATPase beta subunit from yeast or tobacco, cytochrome c1 from yeast), chloroplasts (cytochrome oxidase subunit Va from yeast, small subunit of rubisco from pea), endoplasmic reticulum lumen (protein disulfide isomerase), vacuole (carboxypeptidase Y and proteinase A from yeast, phytohemagglutinin from French bean), peroxisomes (D-aminoacid oxidase, uricase) and lysosomes (hydrolases).

Following transformation, a plant may be regenerated, *e.g.*, from single cells, callus tissue, or leaf discs, as is standard in the art. Almost any plant can be entirely

regenerated from cells, tissues, and organs of the plant. Available techniques are reviewed in Vasil *et al.* (1984) in *Cell Culture and Somatic Cell Genetics of Plants, Vols. I, II, and III, Laboratory Procedures and Their Applications* (Academic press); and Weissbach *et al.* (1989) *Methods for Plant Mol. Biol.*

The transformed plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited, and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

The particular choice of a transformation technology will be determined by its efficiency to transform certain target species, as well as the experience and preference of the person practicing the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant or microalgae plastids is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

Also according to the invention, there is provided a plant or microalgae cell having the constructs of the invention. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector including the construct into a plant cell. For integration of the construct into the plastid genome (the "plastome), such introduction will be followed by recombination between the vector and the plastome genome to introduce the operon sequence of nucleotides into the plastome. RNA encoded by the introduced nucleic acid construct (operon) may then be transcribed in the cell and descendants thereof, including cells in plants regenerated from transformed material. A gene stably incorporated into the plastome of a plant or microalgae is passed from generation to generation to descendants of the plant or microalgae, so such descendants should show the desired phenotype.

The present invention also provides a plant or microalgae culture comprising a plant cell as disclosed. Transformed seeds and plant parts are also encompassed. As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny, meaning descendants, not limited to the immediate generation of descendants but including all generations of

descendants. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to naturally occurring, deliberate, or inadvertent caused mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

In addition to a plant or microalgae, the present invention provides any clone of such a plant or microalgae, seed, selfed or hybrid or mated descendants, and any part of any of these, such as cuttings or seed for plants. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed, and so on. Also encompassed by the invention is a plant or microalgae which is a sexually or asexually propagated off-spring, clone, or descendant of such a plant or microalgae, or any part or propagule of said plant, off-spring, clone, or descendant. Plant or microalgae extracts and derivatives are also provided.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* ssp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidental*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables, ornamentals, and conifers.

Preferably, plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea, and other root, tuber, or seed crops. Important seed crops are oil-seed rape, sugar beet,

maize, sunflower, soybean, and sorghum. Horticultural plants to which the present invention may be applied may include lettuce; endive; and vegetable brassicas including cabbage, broccoli, and cauliflower; and carnations and geraniums. The present invention may be applied to tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper, chrysanthemum, petunia, rose, poplar, eucalyptus, and pine.

Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans including guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

Microalgae include but are not limited to the Chlorophyta and the Rhodophyta and may be such organisms as Chlamydomonas, Haematococcus, and Ouneliella.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. Unless indicated otherwise, the respective contents of the documents cited herein are hereby incorporated by reference to the extent they are not inconsistent with the teachings of this specification.

Percentages and ratios given herein are by weight, and temperatures are in degrees Celsius unless otherwise indicated. The references cited within this application are herein incorporated by reference to the extent applicable. Where necessary to better exemplify the invention, percentages and ratios may be cross-combined.

Example 1: Isolation of Orfs Encoding Enzymes of the Mevalonate Pathway for the Construcion of Vectors pFCO1 and pFCO2

In an exemplified embodiment, vectors containing open reading frames (orfs) encoding enzymes of the mevalonate pathway are constructed. Polynucleotides derived from the yeast Saccharomyces cerevisiae, the plant Arabidopsis thaliana, and the eubacterium Streptomyces sp CL190 are used for the construction of vectors, including

plastid delivery vehicles, containing orfs for biosynthesis of the mevalonate pathway enzymes. Construction of the vectors is not limited to the methods described. It is routine for one skilled in the art to choose alternative restriction sites, PCR primers, etc. to create analogous plasmids containing the same orfs or other orfs encoding the enzymes of the mevalonate pathway. Many of the steps in the construction of the plasmids of the subject invention can utilize the joining of blunt-end DNA fragments by ligation. As orientation with respect to the promoter upstream (5') of the described orfs can be critical for biosynthesis of the encoded polypeptides, restriction analysis is used to determine the orientation in all instances involving blunt-end ligations. A novel directional ligation methodology, chain reaction cloning (Pachuk et al., Gene 243:19-25, 2000), can also be used as an alternative to standard ligations in which the resultant orientation of the insert is not fixed. All PCR products are evaluated by sequence analysis as is well known in the art.

The construction of a synthetic operon comprising three yeast orfs encoding phosphomevalonate kinase, mevalonate kinase, and mevalonate diphosphate decarboxylase is described by Hahn et al. (Hahn et al., J. Bacteriol. 183:1-11, 2001). This same synthetic operon, contained within plasmid pFCO2, is able to synthesize, *in vivo*, polypeptides with enzymatic activities able to convert exogenously supplied mevalonate to IPP as demonstrated by the ability of the mevalonate pathway orfs to complement the temperature sensitive dxs::kanr lethal mutation in E. coli strain FH11 (Hahn et al., 2001).

Plasmids pFCO1 and pFCO2 containing a synthetic operon for the biosynthesis of IPP from mevalonate are constructed as follows: Three yeast orfs encoding mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase are isolated from S. cerevisiae genomic DNA by PCR using the respective primer sets

FH0129-2:

5' GGACTAGTCTGCAGGAGGAGTTTTAATGTCATTACCGTTCTTAAC TTCTGCACCGGG-3' (sense) (SEQ ID NO: 1) and

FH0129-1:

5' TTCTCGAG***CTTAAGAGTAGCAATATTTACCGGAGCAGTTACACTA***

GCAGTATATACAGTCATTAAAACTCCTCCTGTGAAGTCCATGGTAAATTCG 3' (antisense) (SEQ IDNO:2);

FH0211-1:

5' TAGCGGCCGCAGGAGGAGTTCATATGTCAGAGTTGAGAGCCTTC AGTGCCCCAGGG 3' (sense) (SEQ ID NO: 3) and

FH0211-2:

5' TTTCTGCAGTTTATCAAGATAAGTTTCCGGATCTTT 3' (antisense) (SEQ ID NO: 4);

CT0419-1:

5' GGAATTCATGACCGTTTACACAGCATCCGTTACCGCACCCG 3' (sense) (SEQ ID NO:5) and

CT0419-2:

5' GGCTCGAGTTAAAACTCCTCTTCCTTTGGTAGACCAGTCTTTGCG 3' (antisense) (SEQ ID NO: 6).

Primer FH0129-2 includes a SpeI site (underlined). Primer FH0129-1 contains an XhoI site (underlined), an AflII site (double-underlined), and 54 nucleotides (bold italics) corresponding to the 5' end of the yeast orf for mevalonate diphosphate decarboxylase. Following PCR using primers FH0129-1 and FH0129-2, a product containing the orf encoding yeast mevalonate kinase is isolated by agarose gel electrophoresis and GeneClean purified. Following restriction with SpeI-XhoI, the PCR product is inserted into the SpeI-XhoI sites of pBluescript(SK+) (Stratagene, LaJolla, CA) by ligation to create pBRG12. Primers FH0211-1 and FH0211-2 contain a NotI site (underlined) and a PstI site (underlined), respectively. Following PCR using primers FH0211-1 and FH0211-2, a product containing the orf encoding yeast phosphomevalonate kinase is restricted with NotI-PstI, purified by GeneClean, and inserted into pGEM-T Easy (Promega Corp, Madison, WI) by ligation to create pERG8. An orf encoding yeast mevalonate diphosphate decarboxylase is isolated by PCR using primers CT0419-1 and

CT0419-2 and inserted directly into pGEM-T Easy by ligation to create pERG19. Restriction of pERG8 with NotI-PstI yields a 1.4 Kb DNA fragment containing the orf for phosphomevalonate kinase. Restriction of pBRG12 with NotI-PstI is followed by the insertion of the 1.4 Kb NotI-PstI DNA fragment by ligation to create pBRG812 containing the orfs for both phosphomevalonate kinase and mevalonate kinase and the 5' end of the orf for yeast mevalonate diphosphate decarboxylase. Restriction of pERG19 with AflII-XhoI yields a 1.2 Kb DNA fragment containing the 3' end of the orf for yeast mevalonate diphosphate decarboxylase missing in pBRG812. Insertion of the 1.2 Kb AflII-XhoI DNA fragment into pBRG812/AflII-XhoI by ligation yields pFCO1 containing the three yeast mevalonate pathway orfs (Fig. 1). Restriction of pFCO1 with XhoI is followed by treatment with the Klenow fragment of T7 DNA polymerase and dNTPs to create blunt ends. Subsequent restriction of pFCO1/XhoI/Klenow with SacI yields a 3.9 Kb DNA fragment containing the three yeast mevalonate pathway orfs. Following agarose gel electrophoresis and GeneClean purification of the 3.9 Kb DNA fragment, it is inserted into the SmaI-SacI sites of pNGH1-amp (Garrett et al., J. Biol. Chem. 273:12457-12465, 1998) by ligation to create pFCO2 (Fig. 2).

Example 2: Construction of E. coli strain FH11 (JM101/dxs::kan^r/pDX4)

A mutant E. coli strain containing a disruption of the chromosomal dxs gene is constructed as described by Hamilton et al. (Hamilton et al., J. Bacteriol. 171:4617-4622, 1989). The strains are grown at 30° C or 44° C in Luria-Bertani (LB) supplemented with the following antibiotics as necessary; ampicillin (Amp) (50 (g/ml), chloramphenicol (Cam) (30 (g/ml), and kanamycin (Kan) (25 (g/ml). Within phagemid DD92 (F. R. Blattner, University of Wisconsin, Madison, WI) is a 19.8 Kb EcoRI fragment of E. coli genomic DNA containing dxs, the gene for DXP synthase. Following the isolation of the phage from E. coli strain LE392, DD92 is restricted with SphI, and the resultant 6.3 Kb fragment is isolated by agarose gel electrophoresis. GeneClean purification of the SphI fragment and restriction with SmaI yields a 2.0 Kb SphI-SmaI fragment containing E. coli dxs. The 2.0 Kb fragment is purified by GeneClean and inserted by ligation into the SphI-HindII sites of pMAK705, a plasmid containing a temperature-sensitive origin of replication (Hamilton et al., J. Bacteriol. 171:4617-4622, 1989). The resulting plasmid containing wt dxs, pDX4, is restricted with SapI, a unique site located in the middle of

the dxs gene, and the 5'-overhangs are filled in with Klenow and dNTPs. The blunt-ended DNA fragment is purified by GeneClean and treated with shrimp alkaline phosphatase (SAP, USB Corp., Cleveland, OH) according to the manufacturer's instructions. pUC4K (Amersham Pharmacia Biotech, Piscataway, NJ) is restricted with EcoRI, Klenow-treated, and the resulting 1.3 Kb blunt-ended DNA fragment containing the gene for Kan resistance is inserted into the filled-in SapI site of pDX4 by blunt-end ligation to create pDX5 with a disruption in E. coli dxs. Competent E. coli JM101 cells are transformed with pDX5, a pMAK705 derivative containing dxs::kanr, and grown to an optical density (A600) of 0.6 at 30° C. Approximately 10,000 cells are plated out on LB/Cam medium prewarmed to 44° C. The plates were incubated at 44° C, and several of the resulting colonies are grown at 44° C in 4 ml of LB/Cam medium. Four 50 ml LB/Cam cultures are started with 0.5 ml from four of the 4 ml cultures and grown overnight at 30° C. Four fresh 50 ml LB/Cam cultures are started with 100 µl of the previous cultures and grown overnight at 30° C. An aliquot of one of the 50 ml cultures is serially diluted 5 x 105 fold, and 5 µl is plated on LB/Cam medium. Following incubation at 30° C, the resulting colonies are used to individually inoculate 3 ml of LB medium containing Cam and Kan. Twelve LB/Cam/Kan cultures are grown overnight at 30° C and used for plasmid DNA isolation. *E. coli* cells where the disrupted copy of dxs is incorporated into the genome are identified by restriction analysis of the isolated plasmid DNA and verified by sequence analysis of the DNA contained in the plasmids. The E. coli JM101 derivative containing the dxs::kanr mutation is designated FH11 (Hahn *et al.* 2001).

Example 3: Assay Demonstrating Synthesis of IPP from Mevalonic Acid in E. coli

The episomal copy of dxs contained on pDX4 in E. coli strain FH11 is "turned off" at 44° C due to a temperature sensitive origin of replication on the pMAK705 derivative (Hamilton et al., J. Bacteriol. 171:4617-4622, 1989). The inability of FH11 to grow at the restrictive temperature demonstrates that dxs is an essential single copy gene in E. coli (Hahn et al., 2001). A cassette containing three yeast mevalonate pathway orfs is removed from pFCO1 and inserted into pNGH1-Amp to form pFCO2 for testing the ability of the mevalonate pathway orfs to complement the dxs::kanr disruption when FH11 is grown at 44° C on medium containing mevalonate. The utility of strain FH11

as a component of an assay for testing the ability of mevalonate pathway orfs to direct the synthesis of IPP is demonstrated as follows:

Colonies of E. coli strain FH11 transformed with pFCO2 or pNGH1-Amp, the expression vector without an insert, are isolated by incubation at 30° C on LB plates containing Kan and Amp. Four ml LB/Kan/Amp cultures containing either FH11/pFCO2 or FH11/pNGH1-Amp are grown overnight at 30° C. Following a 10,000-fold dilution, 10 µl portions from the cultures are spread on LB/Kan/Amp plates that are prewarmed to 44° C or are at rt. Approximately 1.3 mg of mevalonic acid is spread on each plate used for FH11/pFCO2. The prewarmed plates are incubated at 44° C, and the rt plates are incubated at 30° C overnight.

FH11/pNGH1-amp cells will not grow at the restrictive temperature of 44° C and FH11/pFCO2 cells are unable to grow at of 44° C unless mevalonic acid (50 mg/L) is added to the growth medium thus establishing the ability of the polypeptides encoded by the mevalonate pathway orfs contained in the synthetic operon within pFCO2 to form IPP from mevalonate in vivo (Hahn *et al.*, 2001).

Example 4: Isolation of Mevalonate Pathway Orfs

In a specific, exemplified embodiment, the isolation of orfs, each encoding a polypeptide with either HMG-CoA synthase enzyme activity, HMG-CoA reductase enzyme activity, or acetoacetyl-CoA thiolase enzyme activity, and construction of vectors containing these orfs is as follows: Synthesis of A. thaliana first strand cDNAs is performed utilizing PowerScript™(reverse transcriptase (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. Specifically, a microfuge tube containing 5 µl of A. thaliana RNA (Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH), 1.8 µl poly(dT)15 primer (0.28 µg/µl, Integrated DNA Technologies, Inc.. Coralville, IA), and 6.2 µl DEPC-treated H2O is heated at 70°C for 10 min and then immediately cooled on ice. The mixture is spun down by centrifugation and 4 µl of 5X First-Strand Buffer (Clontech), 2 µ(l Advantage UltraPure PCR dNTP mix (10 mM each, Clontech) and 2 µ(l 100 mM DTT are added and the entire contents mixed by pipetting. Following the addition of 1 µ(l reverse transcriptase (Clontech) and mixing by pipetting, the contents are incubated at 42° C for 90 min and then heated at 70° C for 15 min to terminate the reaction.

The resulting *A. thaliana* first strand cDNAs are used as templates for the synthesis of an orf encoding HMG-CoA synthase and a truncated HMG-CoA reductase by PCR in a Perkin-Elmer GeneAmp PCR System 2400 thermal cycler utilizing the Advantage®-HF 2 PCR Kit (Clontech) according to the manufacturer's instructions. An *A. thaliana* HMG-CoA synthase orf is isolated using the following PCR primers:

1) 5' GCTCTAGATGCGCAGGAGGCACATATGGCGAAGAACGTTGGGATTTTG GCTATGGATATCTATTTCCC 3' (sense) (SEQ ID NO: 7); and

2) 5' CG***CTCGAG***TCGACGGATCCTCAGTGTCCATTGGCTACAGATCCATCTTC ACCTTTCTTGCC 3' (antisense) (SEQ ID NO: 8);

containing the restriction site *Xba*I shown underlined, the restriction site *Xho*I shown in bold italic and the restriction site *Sal*I shown double underlined. Specifically, 2 (l cDNA, 5 µ(l 10X HF 2 PCR Buffer (Clontech), 5 µl 10X HF 2 dNTP Mix (Clontech), 1 µl each of the primers described above, 1 µl 50X Advantage-HF 2 Polymerase Mix (Clontech), and 35 µl PCR-Grade H2O (Clontech) are combined in a 0.5 ml PCR tube. The mixture is heated at 94° C for 15 sec then subjected to 40 PCR cycles consisting of 15 sec at 94° C and 4 min at 68° C. After a final incubation at 68° C for 3 min, the reaction is cooled to 4° C. Agarose gel electrophoresis is performed on a 10 µl aliquot to confirm the presence of a DNA fragment of the predicted size of 1.4 Kb. The PCR is repeated in triplicate to generate enough product for its isolation by gel excision and purification by GeneClean (Qbiogene, Inc., Carlsbad CA). Following restriction with *Xba*I-*Xho*I and purification by GeneClean, the 1.4 Kb PCR product is inserted into the *Xba*I-*Xho*I sites of pBluescript(SK+) by ligation to form putative pBSHMGS constructs. Sequence analysis of several of the candidate constructs is performed to identify inserts with DNA identical to the published *A. thaliana* orf for HMG-CoA synthase and are used for the construction of pBSHMGSR as described below.

An *A. thaliana* orf encoding a polypeptide with HMG-CoA reductase enzyme activity is synthesized by PCR essentially as described above using the following primers:

3) 5' CCGCTCGAGCACGTGGAGGCACATATGCAATGCTGTGAGATGCCT GTTGGATACATTCAGATTCCTGTTGGG 3' (sense) (SEQ ID NO: 9); and

4) 5' GG*GGTACC*TG**CGGCCG**GATCCCGGGTCATGTTGTTGTTGTTGTCGT TGTCGTTGCTCCAGAGATGTCTCGG 3' (antisense) (SEQ ID NO: 10);

containing the restriction site *Xho*I shown underlined, the restriction site *Kpn*I shown in italic, the restriction site *Eag*I shown in bold, and the restriction site *Sma*I shown double underlined. The 1.1 Kb PCR product is isolated by agarose gel electrophoresis, purified by GeneClean and inserted into the pT7Blue-3 vector (Novagen, Inc., Madison, WI) using the Perfectly Blunt™ Cloning Kit (Novagen) according to the manufacturer's instructions. Sequence analysis is performed to identify constructs containing *A. thaliana* DNA encoding the desired C-terminal portion of the published HMG-CoA reductase amino acid sequence and are designated pHMGR.

PCR is performed on *S. cerevisiae* genomic DNA (Invitrogen, Corp., Carlsbad, CA) by using the Advantage®-HF 2 PCR Kit (Clontech) according to the manufacturer's instructions and the following primers:

5) 5' ACAACA*CCGCGGC*GGCCGC**GTCGAC**TACGTAGGAGGCACATATGTC TCAGAACGTTTACATTGTATCGACTGCC 3' (sense) (SEQ ID NO: 11); and

6) 5' GC***TCTAGA***GGATCCTCATATCTTTTCAATGACAATAGAGGAAGCACC ACCACC 3' (antisense) (SEQ ID NO: 12);

containing the restriction site *Not*I shown underlined, the restriction site *Sac*II shown in italic, the restriction site *Sal*I shown in bold, the restriction site SnaBI shown double underlined, and the restriction site *Xba*I in bold italic. The 1.2 Kb PCR product is isolated by agarose gel electrophoresis, purified by GeneClean and inserted into the vector pT7Blue-3 (Novagen,) using the Perfectly Blunt™ Cloning Kit (Novagen) according to the manufacturer's instructions. Sequence analysis is performed to identify constructs containing *S. cerevisiae* DNA identical to the published orf encoding acetoacetyl-CoA thiolase and they are designated pAACT.

Example 5: Construction of pHKO1

In an exemplified embodiment, a pBluescript(SK+) derivative containing an operon with orfs encoding polypeptides with enzymatic activities for HMG-CoA synthase, HMG-CoA reductase, and acetoacetyl-CoA thiolase is constructed as follows: Following restriction of pHMGR with *Xho*I-*Kpn*I, isolation of the 1.1 Kb DNA fragment by agarose gel electrophoresis, and purification by GeneClean, the 1.1 Kb *Xho*I-*Kpn*I DNA fragment containing the orf encoding the C-terminal portion of *A. thaliana* HMG-CoA reductase is inserted into the *Sal*I-*Kpn*I sites of pBSHMGS by ligation to

create pBSHMGSR. Following restriction of pAACT with *Sac*II-*Xba*I, isolation of the 1.2 Kb DNA fragment containing the orf encoding yeast acetoacetyl-CoA thiolase by agarose gel electrophoresis, and purification by GeneClean, the 1.2 Kb *Sac*II-*Xba*I DNA fragment is inserted into the *Sac*II-*Xba*I sites of pBSHMGSR by ligation to create pHKO1 (Fig. 3).

Example 6: Construction of pHKO2

In a specific, exemplified embodiment, a vector containing a synthetic operon consisting of six orfs encoding polypeptides with acetoacetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase enzymatic activities, thus comprising the entire mevalonate pathway, is constructed as follows: Restriction of pHKO1 with *Eag*I yields a 3.7 Kb DNA fragment containing orfs encoding yeast acetoacetyl-CoA thiolase, *A. thaliana* HMG-CoA synthase, and a truncated *A. thaliana* HMG-CoA reductase. Following isolation of the 3.7 Kb *Eag*I DNA fragment by agarose gel electrophoresis and purification by GeneClean, it is directionally inserted into the *Not*I site of pFCO2 (Hahn *et al.*, 2001) utilizing the methodology of chain reaction cloning (Pachuk et al., 2000), thermostable Ampligase((Epicentre Technologies, Madison, WI), and the following bridge oligonucleotide primers:

1) 5' TGGAATTCGAGCTCCACCGCGGTGGCGGCCGCGTCGACGCCGGCGGAG GCACATATGTCT 3'(SEQ ID NO: 13); and

2) 5' AACAACAACAACATGACCCGGGATCCGGCCGCAGGAGGAGTTCATATG TCAGAGTTGAGA 3'(SEQ ID NO: 14);

as follows: Agarose gel electrophoresis is performed on the 8.1 Kb pFCO2/*Not*I DNA fragment and the 3.7 Kb *Eag*I DNA fragment isolated from pHKO1 to visually estimate their relative concentrations. Approximately equivalent amounts of each fragment totaling 4.5 µl, 1 µl of each bridge oligo at a concentration of 200 nM, 5 µl Ampligase® 10X Reaction Buffer (Epicentre), 3 µl Ampligase® (5U/(l) (Epicentre), and 35.5 µl PCR grade H2O are added to a 0.5 ml PCR tube. The mixture is heated at 94° C for 2 min then subjected to 50 PCR cycles consisting of 30 sec at 94° C, 30 sec at 60° C, and 1 min at 66° C. After a final incubation at 66° C for 5 min, the reaction is cooled to 4° C. Colonies resulting from the transformation of *E. coli* strain NovaBlue (Novagen) with 1

μl of the directional ligation reaction are grown in LB medium supplemented with ampicillin at a final concentration of 50 μg/ml. Restriction analysis with NaeI-KpnI of mini-prep plasmid DNA from the liquid cultures is performed to identify candidate pHKO2 constructs by the presence of both a 5.7 and a 6.2 Kb DNA fragment. Further analysis by restriction with *Sma*I-*Xho*I to generate both a 3.9 and 7.9 Kb DNA fragment confirms the successful construction of pHKO2 (Fig. 4).

Example 7:Assay Demonstrating the Synthesis of IPP from Acetyl-CoA in E. coli

In a specific, exemplified embodiment, a derivative of pNGH1-amp (Hahn *et al.*, 2001), containing the entire mevalonate pathway, is assayed (Fig. 5) for its ability to synthesize IPP from endogenous acetyl-CoA in *E. coli* strain FH11, containing the temperature sensitive *dxs*::kan^r knockout (Hahn *et al.*, 2001), as follows: Colonies resulting from the transformation of FH11, by pHKO2, containing orfs encoding polypeptides with enzymatic activities for acetoacetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase, are isolated by incubation at 30° C on LB plates containing Kan and Amp. Several 4 ml LB/Kan/amp samples are individually inoculated with single colonies from the FH11/pHKO2 transformation. Following growth at 30° C overnight, the FH11/pHKO2 cultures are diluted 100,000-fold, and 5 μl aliquots are spread on LB/Kan/amp plates at room temperature (rt) or that are prewarmed to 44° C. The prewarmed plates are incubated at 44° C, and the rt plates are incubated at 30° C overnight. FH11 and FH11/pNGH1amp cells will not grow at the restrictive temperature of 44° C (Hahn *et al.*, 2001). FH11/pHKO2 cells are able to grow at 44° C, thus establishing the ability, of a synthetic operon comprising the entire mevalonate pathway, to form IPP from acetyl-CoA and thereby overcome the dxs::kan^r block to MEP pathway biosynthesis of IPP in *E. coli* strain FH11.

Example 8: Construction of pHKO3

In another exemplified embodiment, a derivative of pBluescript(SK+) containing an operon comprising orfs, which in their summation is the entire mevalonate pathway, is constructed as follows: pHKO1, containing orfs encoding acetoacetyl-CoA thiolase, HMG-CoA synthase, and an N-terminal truncated HMG-CoA reductase, is restricted with

*Sal*I-*Not*I and purified by GeneClean. The pBluescript(SK+) derivative pFCO1, containing the orfs encoding mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase, has been described above in Example 1. Following restriction of pFCO1 with *Xho*I-*Not*I, isolation by agarose gel electrophoresis, and purification by GeneClean, the 3.9 Kb DNA fragment containing the mevalonate pathway orfs is inserted into pHKO1/*Sal*I-*Not*I by directional ligation (Pachuk *et al.*, 2000) utilizing thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the following bridging oligonucleotides:

1) 5' CTCAACTCTGACATATGAACTCCTCCTGCGGCCGCCGCGGTGGAGCTCC AGCTTTTGTTCCC 3' (SEQ ID NO: 15); and

2) 5' GGTCTACCAAAGGAAGAGGAGTTTTAACTCGACGCCGGCGGAGGCACA TATGTCTCAGAACG 3' (SEQ ID NO: 16);

essentially as described for the construction of pHKO2. Restriction analysis is performed with KpnI to confirm the successful construction of pHKO3 (Fig. 6).

Example 9: Construction of Tobacco Plastid Transformation Vector pHKO4

In an exemplified embodiment, a vector containing a *Nicotiana tabacum* plastid pseudogene is utilized to create a plastid transformation vector as follows: The pBluescript(SK+) derivative designated as pBSNT27 (Fig. 7, SEQ ID NO: 17) contains a 3.3 Kb *Bgl*II-*Bam*HI DNA fragment of the *N. tabacum* chloroplast genome corresponding approximately to base-pairs 80553-83810 of the published nucleotide sequence (Sugiura, M., 1986, and Tsudsuki, T., 1998.). A unique restriction site contained within the tobacco *inf*A pseudogene located on pBSNT27 is cleaved with *Bgl*II and the resulting 5' overhangs are filled in with Klenow and dNTPs. The resulting 6.2 Kb blunt-ended DNA fragment is GeneClean purified. Following restriction of pHKO3 with *Eag*I, filling in of the resulting 5' overhangs with Klenow and dNTPs, isolation by agarose gel electrophoresis, and purification by GeneClean, the resulting 7.7 Kb blunt-ended DNA fragment, containing orfs encoding the entire mevalonate pathway, is directionally inserted into the blunt-ended BglII site of pBSNT27 utilizing chain reaction cloning (Pachuk *et al.*, 2000.), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the following bridging oligonucleotides:

1) 5' GATCTTTCCTGAAACATAATTTATAATCAGATCGGCCGCAGGAGGAG

TTCATATGTCAGAGTTGAG 3' (SEQ ID NO: 18); and

2) GACAACAACAACAACATGACCCGGGATCCGGCCGATCTAAACAAACCCG GAACAGACCGTTGGGAA 3' (SEQ ID NO: 19);

to form the tobacco plastid-specific transformation vector pHKO4 (Fig. 8).

Alternatively, other derivatives of pBSNT27 can be constructed, using skills as known in the art, that are not reliant upon an available restriction site(s) in the pseudogene. For example, although the infA pseudogene comprises basepairs 3861-4150 in pBSNT27, there are unique restriction sites in close proximity, upsteam and downstream, that can be utilized to excise the entire pseudogene followed by its replacement with an orf or gene cluster comprising multiple orfs, e.g. the complete mevalonate pathway described above. Specifically, there is a unique BsrGI site at 3708 base pairs and a unique SexAI restriction site at 4433 base pairs within pBSNT27. Thus, as will be readily apparent to those skilled in the art, one can replace the infA pseudogene entirely by inserting a BsrGI- SexAI DNA fragment containing DNA, comprising orfs encoding the entire mevalonate pathway, that is flanked by the excised DNA originally flanking the infA pseudogene, i.e. DNA corresponding to 3708-3860 and 4151-4433 base pairs in pBSNT27. The resultant construct will be missing the pseudogene, but will contain the excised flanking DNA restored to its original position and now surrounding the mevalonate pathway orfs. Also, a similar strategy, that will also be apparent to those skilled in the art in view of this disclosure, can be employed that restores the intact pseudogene to a location between the DNA originally flanking it, yet linked to an orf or orfs located upstream and/or downstream of the pseudogene and adjacent to the original flanking DNA.

Example 10: Construction of Vectors Containing Orfs Encoding IPP Isomerase (pHKO5 and pHKO6)

In a specific, exemplified embodiment, orfs encoding IPP isomerase are isolated and vectors containing an operon comprising orfs for the entire mevalonate pathway and an additional orf for IPP isomerase are constructed as follows: A *Rhodobacter capsulatus* orf encoding a polypeptide with IPP isomerase activity is isolated by PCR from genomic DNA (J. E. Hearst, Lawrence Berkeley Laboratories, Berkeley, CA) using the following primers:

1) 5' CGCTCGAG**TACGTA**AGGAGGCACATATGAGTGAGCTTATACCCGCCTG GGTTGG 3' (sense) (SEQ ID NO: 20); and

2) 5' GC*TCTAGA*GATATCGGATCC**GCGGCCG**CTCAGCCGCGCAGGATCGATCC GAAAATCC 3' (antisense) (SEQ ID NO: 21);

containing the restriction sites *Xho*I shown underlined, *Bsa*AI shown in bold, *Xba*I shown in italic, *Eco*RV shown double underlined, and *Not*I shown in bold italic. The PCR product is restricted with *Xho*I-*Xba*I, isolated by agarose gel electrophoresis, purified by GeneClean, and inserted into the *Xho*I-*Xba*I sites of pBluescript(SK+) by ligation to form pBSIDI. Sequence analysis is performed to identify the plasmids containing *R. capsulatus* DNA identical to the complementary sequence of base pairs 34678-34148, located on contig rc04 (Rhodobacter Capsulapedia, University of Chicago, Chicago, IL). Following restriction of pBSIDI with *Bsa*AI-*Eco*RV, agarose gel electrophoresis and GeneClean purification, the 0.5 Kb *Bsa*AI-*Eco*RV DNA fragment containing the *R. capsulatus* orf is inserted into the dephosphorylated *Sma*I site of pHKO3 by blunt-end ligation to create pHKO5 (Fig. 9). This establishes the isolation of a previously unknown and unique orf encoding *R. capsulatus* IPP isomerase.

A *Schizosaccharomyces pombe* orf encoding a polypeptide with IPP isomerase activity is isolated from plasmid pBSF19 (Hahn and Poulter, J. Biol. Chem. 270:11298-11303, 1995) by PCR using the following primers

3) 5' GCTCTAGA**TACGTA**GGAGGCACATATGAGTTCCCAACAAGAGAAAAA GGATTATGATGAAGAACAATTAAGG 3' (sense) (SEQ ID NO: 22); and

4) 5' CGCTCGAGCCCGGGGGATCCTTAGCAACGATGAATTAAGGTATCTTGG AATTTTGACGC 3' (antisense) (SEQ ID NO: 23);

containing the restriction site *Bsa*AI shown in bold and the restriction site *Sma*I shown double underlined. The 0.7 Kb PCR product is isolated by agarose gel electrophoresis, purified by GeneClean and inserted into the pT7Blue-3 vector (Novagen, Inc., Madison, WI) using the Perfectly Blunt™ Cloning Kit (Novagen) according to the manufacturer's instructions. Sequence analysis is performed to identify constructs containing *S. pombe* DNA identical to the published DNA sequence (Hahn and Poulter, 1995) and are designated pIDI. Following restriction of pIDI with *Bsa*AI-*Sma*I, isolation by agarose gel electrophoresis, and purification by GeneClean, the 0.7 Kb *Bsa*AI-*Sma*I DNA fragment containing the orf encoding *S. pombe* IPP isomerase is inserted into the dephosphorylated

*Sma*I site of pHKO3 by blunt-end ligation to create pHKO6.

Example 11: Construction of Vectors Containing Alternative Orfs for Mevalonate Pathway Enzymes and IPP Isomerase

In another exemplified embodiment, vectors containing open reading frames (orfs) encoding enzymes of the mevalonate pathway and IPP isomerase other than those described above are constructed. Polynucleotides derived from the yeast *Saccharomyces cerevisiae*, the plant *Arabidopsis thaliana*, and the bacteria *Rhodobacter capsulatus* and Streptomyces sp strain CL190 are used for the construction of vectors, including plastid delivery vehicles, containing orfs for biosynthesis of the encoded enzymes. Construction of the vectors is not limited to the methods described. One skilled in the art may choose alternative restriction sites, PCR primers, etc. to create analogous plasmids containing the same orfs or other orfs encoding the enzymes of the mevalonate pathway and IPP isomerase.

Specifically, by way of example, genomic DNA is isolated from Streptomyces sp strain CL190 (American Type Culture Collection, Manassas, VA) using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. An orf encoding a polypeptide with HMG-CoA reductase activity (Takahashi *et al.*, J. Bacteriol. 181:1256-1263, 1999) is isolated from the Streptomyces DNA by PCR using the following primers :

1) 5' CCGCTCGAGCACGTGAGGAGGCACATATGACGGAAACGCACGCCATAG CCGGGGTCCCGATGAGG 3' (sense) (SEQ ID NO: 24); and

2) 5' GG*GGTACC***GCGGCCGC**ACGCGTCTATGCACCAACCTTTGCGGTCTT GTTGTCGCGTTCCAGCTGG 3' (antisense) (SEQ ID NO: 25);

containing the restriction site *Xho*I shown underlined, the restriction site *Kpn*I shown in italics, the restriction site *Not*I shown in bold, and the restriction site *Mlu*I shown double underlined. The 1.1 Kb PCR product is isolated by agarose gel electrophoresis, purified by GeneClean and inserted into the pT7Blue-3 vector (Novagen, Inc., Madison, WI) using the Perfectly Blunt™ Cloning Kit (Novagen) according to the manufacturer's instructions. Sequence analysis is performed to identify constructs containing Streptomyces sp CL190 DNA identical to the published sequence and are designated pHMGR2.

Alternatively, using skills as known in the art, an orf encoding a truncated *S. cerevisiae* HMG-CoA reductase (Chappel et al., US patent 5,349,126 1994) can be isolated by PCR and inserted into pT7Blue-3 (Novagen, Inc., Madison, WI) to construct a vector for use in building a gene cluster comprising the entire mevalonate pathway, in an analgous fashion to the use of the Streptomyces sp CL190 orf encoding HMG-CoA reductase, as described herein.

Following restriction of pAACT (see Example 4) with *Sac*II-*Xba*I, isolation of the 1.2 Kb DNA fragment containing the orf encoding yeast acetoacetyl-CoA thiolase by agarose gel electrophoresis, and purification by GeneClean, the 1.2 Kb *Sac*II-*Xba*I DNA fragment is inserted into the *Sac*II-*Xba*I sites of pBSHMGS (see Example 4) by ligation to create pBSCTGS. Following restriction of pHMGR2 with *Xho*I-*Kpn*I, isolation of the 1.1 Kb DNA fragment by agarose gel electrophoresis, and purification by GeneClean, the 1.1 Kb *Xho*I-*Kpn*I DNA fragment containing the orf encoding *Streptomyces* sp CL190 HMG-CoA reductase is inserted into the *Xho*I-*Kpn*I sites of pBSCTGS by ligation to create the pBluescript(SK+) derivative, pFHO1 (Fig. 10).

A derivative of pFHO1 containing an operon with orfs, which in their summation comprise the entire mevalonate pathway, is constructed as follows: pFHO1 is restricted with *Sna*BI and the resulting 6.6 Kb blunt-ended DNA fragment is purified by GeneClean. Following the restriction of pFCO1 (see Example 1) with *Not*I-*Xho*I, the resulting 3.9 Kb DNA fragment is isolated by agarose gel electrophoresis and purified by GeneClean. The 5' overhangs of the 3.9 Kb DNA fragment are filled in with Klenow and dNTPs. Following purification by GeneClean, the blunt-ended DNA fragment containing three mevalonate pathway orfs (Hahn *et al.*, 2001) is inserted into the *Sna*BI site of pFHO1 utilizing directional ligation methodology (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the bridging oligonucleotides:

3) 5' GAGCTCCACCGCGGCGGCCGCGTCGACTACGGCCGCAGGAGGAGTTCA TATGTCAGAGTT 3' (SEQ ID NO: 26); and

4) 5' TCTACCAAAGGAAGAGGAGTTTTAACTCGAGTAGGAGGCACATATGTC TCAGAACGTTTA 3' (SEQ ID NO: 27);

to form pFHO2 (Fig. 11).

A derivative of pFHO2 containing an operon with orfs, which in their summation comprise the entire mevalonate pathway and an orf encoding IPP isomerase is constructed

as follows: pFHO2 is restricted with *Mlu*I and the resulting 5' overhangs are filled in with Klenow and dNTPs. The 10.6 Kb blunt-ended DNA fragment is purified by GeneClean. Following restriction of pBSIDI with *Bsa*AI-*Eco*RV, agarose gel electrophoresis and GeneClean purification, the resulting blunt-ended 0.5 Kb DNA fragment containing the *R. capsulatus* IPP isomerase orf is inserted into the filled in *Mlu*I site of pFHO2 utilizing directional ligation methodology (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the following bridging oligonucleotides:

5) 5' CAAGACCGCAAAGGTTGGTGCATAGACGCGGTAAGGAGGCACATATGA GTGAGCTTATAC 3' (SEQ ID NO: 28); and

6) 5' CCTGCGCGGCTGAGCGGCCGCGGATCCGATCGCGTGCGGCCGCGGTACC CAATTCGCCCT 3'(SEQ ID NO: 29);

to form pFHO3 (Fig. 12).

Following the restriction of pBluescript(SK+) with SacII-*Xba*I and purification by GeneClean, a 1.3 Kb *Sac*II-*Xba*I DNA fragment containing the orf encoding *S. cerevisiae* acetoacetyl-CoA thiolase, isolated from pAACT (see Example 4) by restriction and agarose gel electrophoresis, is inserted into pBluescript(SK+)/*Sac*II-*Xba*I by ligation. The resulting plasmid, pBSAACT, is restricted with *Xba*I, treated with Klenow and dNTPs, and purified by GeneClean. Following restriction of *Streptomyces* sp CL190 genomic DNA with *Sna*BI, a blunt-ended 6.8 Kb DNA fragment, containing five (5) orfs encoding polypeptides with HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase and IPP isomerase enzymatic activities (Takagi *et al.*, J. Bacteriol. 182:4153-4157, 2000 and Kuzuyama *et al.*, Proc. Natl. Acad. Sci. USA 98:932-7, 2001), is isolated by agarose gel electrophoresis, purified by GeneClean and inserted into the filled in *Xba*I site of pBSAACT utilizing directional ligation methodology (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the bridging oligonucleotides:

7) 5' TGTCATTGAAAAGATATGAGGATCCTCTAGGTACTTCCCTGGCGTGTGC AGCGGTTGACG 3' (SEQ ID NO: 30); and

8) 5' CGATTCCGCATTATCGGTACGGGTGCCTACCTAGAACTAGTGGATCCCC CGGGCTGCAGG 3' (SEQ ID NO: 31);

to form pFHO4 (Fig 13). Transformation experiments to isolate pFHO4 constructs are performed with *E. coli* competent cells utilizing media containing ampicillin.

Alternatively, media containing only fosmidomycin (20 μg/ml) as the selection agent is used for the direct isolation of pFHO4 constructs containing the *Streptomyces* sp CL190 gene cluster.

The construction of vectors pHKO2, pHKO3, pHKO5, pHKO6, pFHO2, pFHO3, and pFHO4, illustrates the many ways of combining orfs isolated from a variety of organisms to encode polypeptides such that in their summation they comprise the entire mevalonate pathway or comprise the entire mevalonate pathway and IPP isomerase.

Example 12: Construction of Tobacco Plastid Transformation Vectors pHKO7 and pHKO8

In a specific, exemplified embodiment, tobacco plastid-specific transformation vectors containing orfs, which in their summation comprise the mevalonate pathway, and an additional orf encoding IPP isomerase are constructed as follows: Restriction of pHKO5 with *Not*I generates a DNA fragment containing six orfs comprising the entire mevalonate pathway and an additional orf encoding *R. capsulatus* IPP isomerase. Restriction of pHKO6 with *Eag*I generates a DNA fragment containing the six orfs comprising the complete mevalonate pathway and an additional orf encoding *S. pombe* IPP isomerase. Following isolation by agarose gel electrophoresis and purification by GeneClean, the 8.2 Kb NotI DNA fragment from pHKO5 is blunt-ended with Klenow and dNTPs and inserted into the blunt-ended *Bgl*II site of pBSNT27 utilizing chain reaction cloning (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the following bridging oligonucleotides:

1) 5' CTTTCCTGAAACATAATTTATAATCAGATCGGCCGCAGGAGGAGTTCA TATGTCAGAGTT 3' (SEQ ID NO: 32); and

2) 5'TTCGGATCGATCCTGCGCGGCTGAGCGGCCGATCTAAACAAACCCGGA ACAGACCGTTGG 3' (SEQ ID NO: 33);

to create the plastid delivery vehicle pHKO7 (Fig. 14) containing orfs encoding the entire mevalonate pathway and an orf encoding *R. capsulatus* IPP isomerase. Following isolation by agarose gel electrophoresis and purification by GeneClean, the 8.4 Kb *Eag*I DNA fragment from pHKO6 is blunt-ended with Klenow and dNTPs and inserted into the blunt-ended *Bgl*II site of pBSNT27 utilizing chain reaction cloning (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the

following bridging oligonucleotides:

3) 5' CTTTCCTGAAACATAATTTATAATCAGATCGGCCGCAGGAGGAGTTCA TATGTCAGAGT 3' (SEQ ID NO: 34); and

4) 5' TCGTTGCTAAGGATCCCCCGGGATCCGGCCGATCTAAACAAACCCGGA ACAGACCGTTGG 3' (SEQ ID NO: 35);

to create the plastid delivery vehicle pHKO8 containing orfs encoding the entire mevalonate pathway plus the *S. pombe* IPP isomerase orf.

Alternatively, either of the IPP isomerase orfs described above can be solely inserted, without orfs for the mevalonate pathway, directly into pBSNT27 (or into any suitable plant transformation vector, known in the art), using skills known in the art.

Example 13: Construction of Vectors used for Increasing Carotenoid Production (pHKO9, pHK10, pHK11, pHK12, and pHK13)

In yet another exemplified embodiment, a derivative of pTrcHisB (Invitrogen) containing a synthetic operon comprising orfs, which in their summation is the entire mevalonate pathway, is constructed as follows: A unique *Not*I site was inserted into pTrcHisB utilizing the following oligonucleotides:

1) 5' CATGGCGGCCGCG 3' (SEQ ID NO: 36); and

2) 5' GATCCGCGGCCGC 3' (SEQ ID NO: 37);

that upon annealing, form a double-stranded DNA linker containing *Not*I with 5' overhangs compatible with *Sty*I and *Bam*HI. Following restriction of pTrcHisB with *Sty*I-*Bam*HI, isolation of the resulting 4.3 Kb DNA fragment by agarose gel electrophoresis, and its purification by GeneClean, the *Not*I linker was inserted into pTrcHisB/*Sty*I-*Bam*HI by ligation. Restriction analysis with *Bsa*AI-*Not*I confirms the successful construction of pTrcHisB-*Not*I (pTHBN1) by the presence of both 2.5 and 1.8 Kb DNA fragments. Following restriction of pHKO3 with *Eag*I, the 7.7 Kb DNA fragment, containing the six mevalonate pathway orfs, is isolated by agarose gel electrophoresis, purified by GeneClean, and inserted into the *Not*I site of pTHBN1 utilizing directional ligation methodology (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the bridging oligonucleotides:

3) 5' TTAAATAAGGAGGAATAAACCATGGCGGCCGCAGGAGGAGTTCATAT GTCAGAGTTGAGA 3' (SEQ ID NO: 38); and

4) 5' AACAACAACAACATGACCCGGGATCCGGCCGCGATCCGAGCTCGAGA TCTGCAGCTGGTA 3' (SEQ ID NO: 39);

to form pHKO9 (Fig. 15).

Derivatives of pTHBN1 containing the entire mevalonate pathway plus an additional orf encoding IPP isomerase are constructed as follows: Following restriction of pHKO5 with *Not*I, the 8.2 Kb DNA fragment, containing the six mevalonate pathway orfs plus an orf encoding *R. capsulatus* IPP isomerase, is isolated by agarose gel electrophoresis, purified by GeneClean, and inserted into the *Not*I site of pTHBN1 utilizing directional ligation methodology (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the bridging oligonucleotides:

5) 5' TCGATTAAATAAGGAGGAATAAACCATGGCGGCCGCAGGAGGAGTTCA TATGTCAGAGTT 3' (SEQ ID NO: 40); and

6) 5' GATTTTCGGATCGATCCTGCGCGGCTGAGCGGCCGCGATCCGAGCTCG AGATCTGCAGCT 3' (SEQ ID NO: 41);

to form pHK10 (Fig. 16). Following restriction of pHKO6 with *Eag*I, the 8.4 Kb DNA fragment, containing the six mevalonate pathway orfs plus an orf encoding *S. pombe* IPP isomerase, is isolated by agarose gel electrophoresis, purified by GeneClean, and inserted into the *Not*I site of pTHBN1 utilizing directional ligation methodology (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the following bridging oligonucleotides:

7) 5' TCGATTAAATAAGGAGGAATAAACCATGGCGGCCGCAGGAGGAGTTCA TATGTCAGAGTT 3' (SEQ ID NO: 42); and

8) 5' TTCATCGTTGCTAAGGATCCCCCGGGATCCGGCCGCGATCCGAGCTCG AGATCTGCAGCT 3' (SEQ ID NO: 43);

to form pHK11.

Derivatives of pTHBN1 containing only an orf encoding IPP isomerase are constructed as follows: pTHBN1 is restricted with *Not*I and the resulting 5' overhangs are filled in with Klenow and dNTPs. The 4.3 Kb pTHBN1/*Not*I blunt-ended DNA fragment is GeneClean purified. Following restriction of pBSIDI with *Bsa*AI-*Eco*RV, agarose gel electrophoresis and GeneClean purification, the resulting blunt-ended 0.5 Kb DNA fragment containing the *R. capsulatus* IPP isomerase orf is inserted into the filled in *Not*I site of pTHBN1 utilizing chain reaction cloning (Pachuk *et al.*, 2000),

thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the following bridging oligonucleotides:

9) 5' TTAAATAAGGAGGAATAAACCATGGCGGCCGTAAGGAGGCACATATG AGTGAGCTTATAC T 3' (SEQ ID NO: 44); and

10) 5' GCCTGCGCGGCTGAGCGGCCGCGGATCCGATGGCCGCGATCCGAGCTC GAGATCTGCAGCT 3' (SEQ ID NO: 45);

to form pHK12. Following restriction of pIDI with *Bsa*AI-*Sma*I, agarose gel electrophoresis and GeneClean purification, the resulting blunt-ended 0.7 Kb DNA fragment containing the *S. pombe* IPP isomerase orf is inserted into the filled in *Not*I site of pTHBN1 utilizing chain reaction cloning (Pachuk *et al.*, 2000), thermostable Ampligase® (Epicentre Technologies, Madison, WI), and the bridging oligonucleotides:

11) 5' TTAAATAAGGAGGAATAAACCATGGCGGCCGTAGGAGGCACATATGA GTTCCCAACAAGA 3' (SEQ ID NO: 46); and

12) 5' ACCTTAATTCATCGTTGCTAAGGATCCCCCGGCCGCGATCCGAGCTCG AGATCTGCAGCT 3' (SEQ ID NO: 47);

to form pHK13.

Example 14: Increased Isoprenoid Production in Cells Containing the MEP Pathway

In another exemplified embodiment, a carotenoid producing *E. coli* strain is utilized to demonstrate the effect of the insertion of orfs encoding the entire mevalonate pathway, or orfs encoding the entire mevalonate pathway and IPP isomerase, or an orf encoding just IPP isomerase, on production of lycopene as follows: Following the transformation of *E. coli* TOP10 F' (Invitrogen) with pAC-LYC (Cunningham *et al.*, J. Bacteriol. 182:5841-5848, 2000), transformed cells are isolated on LB/Cam (30 µg/ml) plates grown at 30° C. TOP10 F'/pAC-LYC competent cells are prepared by the $CaCl_2$ method (Sambrook *et al.*, 1989) following growth in LB/Cam in darkness at 28° C and 225 rpm to an optical density (A_{600}) of 0.6. Competent TOP10 F'/pAC-LYC cells are transformed with one of the following plasmids: pTrcHisB; pHKO9, a pTrcHisB derivative containing the entire mevalonate pathway; pHK10, a pTrcHisB derivative containing the entire mevalonate pathway plus the orf encoding *R. capsulatus* IPP isomerase; pHK11, a pTrcHisB derivative containing the entire mevalonate pathway plus the orf encoding *S. pombe* IPP isomerase; pHK12, a pTrcHisB derivative containing the

orf encoding *R. capsulatus* IPP isomerase; and pHK13, a pTrcHisB derivative containing the orf encoding *S. pombe* IPP isomerase. The bacterial strains described above, comprising pTHBN1 derivatives containing the mevalonate pathway orfs and/or an orf encoding IPP isomerase, are designated HK1, HK2, HK3, HK4, and HK5 respectively. The resulting transformants are isolated as colonies from LB/Cam/amp plates grown at 30° C. Single colonies of TOP10 F'/pAC-LYC/pTrcHisB and HK1 (TOP10 F'/pAC-LYC/pHKO9) are used to individually inoculate 4 ml LB/Cam/amp cultures and grown overnight in the dark at 28° C and 225 rpm. The cultures are serially diluted 10,000 to 100,000-fold, plated on LB/Cam/amp medium containing IPTG, and grown in the dark at rt for 2 to 10 days. The plates are visually examined for an increase in lycopene production as evident by a "darkening" of the light pink colored colonies that are present on the control plates corresponding to TOP10 F'/pAC-LYC/pTrcHisB. The same experiments are performed with strains HK2, HK3, HK4 , and HK5 to determine, visually, the effect of the orfs contained within pHK10, pHK11, pHK12, and pHK13 on lycopene production in TOP10 F'/pAC-LYC cells. The quantification of the carotenoid lycopene in cells, identified as potential overproducers due to their darker color when compared to the color of TOP10 F'/pAC-LYC/pTHBN1 cells, is performed utilizing a spectrophotometric assay as described by Cunningham *et al.* (Cunningham *et al.*, 2000). Increased production of lycopene in *E. coli* cells containing the entire mevalonate pathway or the entire mevalonate pathway plus an additional orf for IPP isomerase establishes that the presence in cells of an additional biosynthetic pathway for the formation of IPP or IPP and DMAPP enhances the production of isoprenoid compounds, such as carotenoids, that are derived from IPP and DMAPP.

Example 15: Demonstration of Antibiotic Resistance Due to the Mevalonate Pathway in MEP Pathway Dependent Cells

In still another exemplified embodiment, *E. coli* cells are transformed with DNA containing orfs, which in their summation comprise the entire mevalonate pathway, and the resulting cells are tested for resistance to the antibiotic fosmidomycin as follows: Following the separate transformation of E. coli TOP10 F' (Invitrogen) with pHKO2, pHKO3 and pHKO9, transformed cells are isolated on LB/Amp (50 μg/ml) plates grown at 30° C. Single colonies of TOP10 F'/pHKO2 (designated strain HK6), TOP10

F'/pHKO3 (designated strain HK7), and TOP10 F'/pHKO9 (designated strain HK8), are used to individually inoculate 4 ml LB/amp cultures and grown overnight at 30° C, 225 rpm. The HK6 and HK7 cultures are serially diluted 10,000 to 100,000-fold and plated on LB containing fosmidomycin (20 μg/ml). The HK8 cultures are serially diluted 10,000 to 100,000-fold and plated on LB/ IPTG containing fosmidomycin (20 μg/ml) Controls are performed with cells comprising TOP10 F' transformed with the parent vectors of pHKO2, pHKO3 and pHKO9, by plating on the appropriate medium containing fosmidomycin establishing that *E. coli* control cells are unable to grow on medium containing fosmidomycin. The ability of transformed *E. coli* cells to grow in the presence of the antibiotic fosmidomycin establishes that the inserted DNA, comprising the entire mevalonate pathway and thus an alternative biosynthetic route to IPP, is functional and can circumvent the inhibition of an enzyme in the trunk line of the MEP pathway.

Example 16: Construction of Plastid Transformation Vectors

In a specific, exemplified embodiment, a plant plastid transformation vector containing a synthetic operon comprising orfs, which in their summation is the entire mevalonate pathway, is constructed as follows: Plasmid pHKO3, a pBluescript derivative containing all six mevalonate pathway orfs, is assembled by restriction of pFCO1 to yield a 3.9 Kb NotI-XhoI DNA fragments containing three mevalonate orfs and its subsequent insertion into the SalI-NotI sites of pHKO1 by directional ligation as described above in Example 8. The plastid transformation vehicle, pHK14 containing the entire mevalonate pathway is constructed as follows: Plastid vector pGS104 (Serino and Maliga, Plant J. 12:687-701, 1997) is restricted with *Nco*I-*Xba*I and the two resulting DNA fragment are separated by agarose gel electrophoresis. Following isolation of the larger DNA fragment by gel excision and its purification by GeneClean, the NcoI-*Xba*I 5' overhangs are dephosphorylated using SAP and filled in with Klenow and dNTPs. The resulting blunt-ended, dephosphorylated DNA fragment derived from pGS104 is GeneClean purified. Following restriction of pHKO3 with EagI, isolation by agarose gel electrophoresis, and purification by GeneClean, the 7.7 Kb DNA fragment is treated with Klenow and dNTPs to fill in the 5' overhangs. The resulting blunt-ended DNA fragment containing the mevalonate pathway is purified by GeneClean and inserted into the

dephosphorylated, Klenow-treated NcoI-XbaI sites of pGS104 by blunt-end ligation to yield pHK14.

Derivatives of pGS104 containing the entire mevalonate pathway plus an additional orf encoding IPP isomerase are constructed as follows: Following restriction of pHKO5 with *Not*I and treatment with Klenow and dNTPs, the resulting 8.2 Kb blunt-ended DNA fragment, containing the six mevalonate pathway orfs plus an orf encoding *R. capsulatus* IPP isomerase, is isolated by agarose gel electrophoresis, purified by GeneClean, and inserted into the dephosphorylated, filled in *Nco*I-XbaI sites of pGS104 by blunt-end ligation to yield pHK15. Following restriction of pHKO6 with EagI and treatment with Klenow and dNTPs, the resulting 8.4 Kb blunt-ended DNA fragment, containing the six mevalonate pathway orfs plus an orf encoding *S. pombe* IPP isomerase, is isolated by agarose gel electrophoresis, purified by GeneClean, and inserted into the dephosphorylated, filled in NcoI-*Xba*I sites of pGS104 by blunt-end ligation to yield pHK16.

Derivatives of pGS104 containing only an orf encoding IPP isomerase are constructed as follows: Following restriction of pBSIDI with *Bsa*AI-*Eco*RV, agarose gel electrophoresis and GeneClean purification, the resulting blunt-ended 0.5 Kb DNA fragment containing the *R. capsulatus* IPP isomerase orf is inserted into the dephosphorylated, filled in *Nco*I-*Xba*I sites of pGS104 by blunt-end ligation to yield pHK17. Following restriction of pIDI with *Bsa*AI-SmaI, agarose gel electrophoresis and GeneClean purification, the resulting blunt-ended 0.7 Kb DNA fragment containing the *S. pombe* IPP isomerase orf is inserted into the dephosphorylated, filled in *Nco*I-*Xba*I sites of pGS104 by blunt-end ligation to yield pHK18.

Example 17: Construction of Transplastomic Plants Containing Orfs Encoding the Mevalonate Pathway or Orfs Encoding the Mevalonate Pathway Coupled with IPP Isomerase

In another exemplified embodiment, tobacco is engineered at the plastid level by using any of the plastid transformation vectors described above, or their equivalents, such as variants of those plastid transformation vectors as can be routinely constructed by means known in the art and containing the orfs as taught and described above. Specifically, *Nicotiana tabacum* var. 'Xanthi NC' leaf sections (1 x 0. 5 cm strips from

in vitro plants with 3 to 5 cm long leaves) are centered in the dish, top side up and bombarded with 1 μm gold micro particles (Kota *et al.*, 1999) coated with DNA containing orfs, which in their summation comprise the entire mevalonate pathway, using a PDS 1000 He device, at 1100 psi. Toxicity is evident in tobacco after three weeks of growth on medium containing the antibiotic fosmidomycin at a concentration of at least 500 micromolar. Transplastomic plants are recovered from leaf sections cultured under lights on standard RMOP shoot regeneration medium or on a Murashige-Skoog salts shoot regeneration medium with 3% sucrose, Gamborg's B5 vitamins, 2 mg/L 6-benzylamino-purine and Phytagel (2.7 g/L), containing 500 μM fosmidomycin for the direct selection of insertion of the entire mevalonate pathway into plastids. Alternatively, the regeneration medium contains an antibiotic, e.g. spectinomycin, for selection based on antibiotic resistance due to any co-transformed gene on the transforming DNA vector, as would be readily apparent to the skilled artisan. De novo green leaf tissue is visible after three weeks. Tissue is removed to undergo a second round of selection on shoot regeneration medium with 500 μM fosmidomycin to encourage homoplasmy and plants are rooted. Genomic DNA is isolated from T0 leaf tissue or T1 leaf tissue derived from in vitro germinated transplastomic seeds utilizing the DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions and is subjected to analysis as is known in the art to confirm homoplasmy. The ability to select directly for a transformation event corresponding to the successful insertion of the mevalonate pathway orfs into plastids establishes the use of orfs, which in their summation comprise the entire mevalonate pathway, as a selectable marker for plastid transformation. The construction of fosmidomycin resistant plants establishes the ability of the mevalonate pathway, when functioning in plant plastids, to provide an alternate biosynthetic route to IPP, thus overcoming the effect of an inhibitor targeting an enzyme in the trunk line of the MEP pathway.

Example 18: Metabolic engineering in transplastomic Solanaceae plants

In another exemplified embodiment, Solanaceae species are engineered at the plastid level using infA pseudogene insertion of a selectable marker and orfs for expression. Specifically, leaf sections of a genetically defined white petunia (or other petunia), are engineered, as for the Solanaceous species tobacco (see Example 16), using

vectors pHK04 or pHKO7, or their equivalents, for insertion of orfs encoding the entire mevalonate pathway or orfs encoding the entire mevalonate pathway and IPP isomerase. Transplastomic Solanaceae plants containing orfs encoding the entire mevalonate pathway and IPP isomerase, and containing an additional orf encoding phytoene synthase, are created by insertion of a pBSNT27 (see Example 9) derived vector, constructed as follows:

A Rhodobacter capsulatus orf encoding a polypeptide with phytoene synthase activity is isolated by PCR from genomic DNA using the primers

1) 5' GCGATATCGGATCCAGGAGGACCATATGATCGCCGAAGCGGATATGGA GGTCTGC 3' (sense) (SEQ ID NO: 65)

2) 5' GCGATATCAAGCTTGGATCCTCAATCCATCGCCAGGCCGCGGTCGCGC GC 3' (antisense) (SEQ ID NO: 66)

containing the restriction site BamHI shown underlined. The 1.1 Kb PCR product is isolated by agarose gel electrophoresis, purified by GeneClean and inserted into the pT7Blue-3 vector (Novagen) using the Perfectly Blunt(Cloning Kit (Novagen) according to the manufacturer's instructions. Sequence analysis is performed to identify constructs containing R. capsulatus DNA identical to the published DNA sequence (SEQ ID NO: 71) and are designated pPHS. Following restriction of pPHS with BamHI, isolation by agarose gel electrophoresis, and purification by GeneClean, the 1.1 Kb BamHI DNA fragment containing the orf encoding R. capsulatus phytoene synthase is inserted into the BglII site of pBSNT27 utilizing chain reaction cloning (Pachuk et al., 2000), thermostable Ampligase((Epicentre Technologies, Madison, WI), and the bridging oligonucleotides

3) 5' CTTTCCTGAAACATAATTTATAATCAGATCCAGGAGGACCATATGA TCGCCGAAGCGGAT 3' (SEQ ID NO: 67); and

4) 5' CGACCGCGGCCTGGCGATGGATTGAGGATCTAAACAAACCCGGAA CAGACCGTTGGGAAG 3' (SEQ ID NO: 68);

to create plastid transformation vector pFHO5. Following restriction of pFHO5 with XcmI, a unique site in the infA pseudogene, and purification by GeneClean, the resulting 3' overhangs are removed by treatment with Mung Bean nuclease and the resulting blunt-ended DNA fragment is purified by GeneClean. Vector pFHO3 is restricted with NotI and the resulting 8.3 Kb DNA fragment, containing Operon E, is isolated by agarose gel electrophoresis and purified by GeneClean. The 5' overhangs of the isolated DNA

fragment are filled in with Klenow and dNTPs and the resulting blunt end DNA fragment, containing Operon E, is inserted into the Mung Bean nuclease treated XcmI site of pFHO5 utilizing chain reaction cloning (Pachuk *et al.*, 2000), thermostable Ampligase((Epicentre Technologies, Madison, WI), and the bridging oligonucleotides

5) 5' ATTTTTCATCTCGAATTGTATTCCCACGAAGGCCGCGTCGACTACG GCCGCAGGAGGAGT3' (SEQ ID NO: 69); and

6) 5' TTCGGATCGATCCTGCGCGGCTGAGCGGCCGGAATGGTGAAGTTG AAAAACGAATCCTTC3' (SEQ ID NO: 70);

to create the plastid transformation vector pFHO6 (Fig. 17).

Alternatively, an orf encoding IPP isomerase can be inserted into the XcmI site of pFHO5, utilizing skills as known in the art, to create a plastid transformation vector containing both an orf encoding phytoene synthase and an orf encoding IPP isomerase. Another alternative uses the infA pseudogene as an insertion site for orfs, encoding phytoene synthase, and/or IPP isomerase, and/or the entire mevalonate pathway, linked with the aadA gene as is known in the art for selection of transplastomic plastids on 500 microgram per liter spectinomycin.

The BioRad PDS 1000 He gene gun is used to deliver BioRad tungsten M10 (0.7 micron approx.) microspheres into petunia (Petunia hybrida 'Mitchell') leaves positioned top-side up. Intact leaves, or equivalent tissues of about 6-8 cm^2 per sample are plated onto shoot regeneration medium consisting of Murashige and Skoog basal medium, B5 vitamins, 3% sucrose, 0.7% (w/v) agar and 3 mg/l BA (6-benzylamino-purine), 0.1 mg/l IAA (Deroles and Gardner, Plant Molec. Biol. 11: 355-364, 1988) in 100 x 10 mm plastic Petri dishes. Leaves are centered in the target zone of the gene gun for bombardment at 1100 psi, third shelf from bottom, ~ 5.6 cm gap, 28 mgHg vacuum. M10 microspheres are coated with DNA using standard procedures of $CaCl_2$ and spermidine precipitation, 1.5 to 2 ug DNA/bombardment. After bombardment, tissues are cultured in light in the presence of antibiotic (500 micromolar fosmidomycin). Each leaf sample is then cut into about 6 pieces and cultured on petunia shooting medium containing 500 micromolar fosmidomycin for 3 to 8 weeks, with subculture onto fresh medium every three weeks. Any green shoots are removed and leaves plated onto the same medium containing 500 micromolar fosmidomycin. Plantlets with at least four leaves and of solid green color (no bleaching on petioles or whorls) are transferred for rooting onto solidified hormone-free

Murashige and Skoog salts with B5 vitamins and 2% sucrose and are grown to flowering. The dependency of increased carotenoid production in Solanacae on the combination of the orfs inserted, be it an orf encoding phytoene synthase alone; or orfs encoding the entire mevalonate pathway and phytoene synthase; or orfs encoding phytoene synthase, the entire mevalonate pathway and IPP isomerase; or orfs for phytoene synthase and IPP isomerase, establishes that the addition of the mevalonate pathway and/or IPP isomerase to plant plastids enhances the production of isoprenoid compounds that are derived from IPP and DMAPP; and the suitability of a pseudogene insertion site for creating transplastomic Petunia.

Example 19: Transformation of microalgae

In a specific exemplified embodiment, chloroplast transformants are obtained by microprojectile bombardment of Chlamydomonas reinhardtii cells and subsequent selection on fosmidomycin. Specifically, a genecluster containing the complete mevalonate pathway is substituted, as a selectable marker, for the coding sequence of the aadA gene in the pUC18 derived vector containing 5-atpA:aadA:rbcL-3 (Goldschmidt-Clermont M., Nucleic Acids Res. 19:4083-4089, 1991) as follows: Plasmid pUC-atpX-AAD is restricted with NcoI, purified by GeneCleanand treated with Mung Bean nuclease to remove the resulting 5' overhangs. Following GeneClean purification, the blunt ended DNA fragment is restricted with HindIII to remove the aadA orf and the remaining DNA fragment, containing approximately 653 base pairs of the C. reinhardtii atpA gene and approximately 437 base pairs of the C. reinhardtii rbcL gene (Goldschmidt-Clermont M., 1991), is isolated by agarose gel electrophoresis and purified by GeneClean. Plasmid pFHO4 is restricted with NdeI, purified by GeneClean, and the resulting 5 overhangs are filled in with Klenow and dNTPs. Following GeneClean purification, the blunt ended DNA fragment is restricted with HindIII and the resulting DNA fragment, containing Operon F (see Fig. 13), is isolated by agarose gel electrophoresis and purified by GeneClean. The blunt end-HindIII fragment is inserted into the blunt end HindIII sites of the DNA fragment isolated from pUC-atpX-AAD by ligation resulting in the orf encoding S. cerevisiae acetoacetylCoA thiolase, located at the beginning of Operon F, to be in frame with the ATG start codon of the 5atpA DNA in pUC-atpX-AAD (Goldschmidt-Clermont M., 1991). The resulting modified yeast orf

only encodes 2 extra amino acids, Met and Ser, appended to the N-terminal Met of the acetoacetylCoA thiolase polypeptide encoded by Operon F. The resulting chlamydomonas plastid transformation vector is designated pHK19. About 10,000 cells are spread on TAP plates containing 200 micromolar fosmidomycin , plates are dried, and then cells are immediately bombarded with M10 or 1 micron gold particles coated with about 2 micrograms of plasmid DNA using the PDS-1000 He gene gun , 1100 psi, fourth shelf from bottom, ~ 2 cm gap, ~28 mgHg vacuum (alternatively cells are spread over a Nytran nylon 0.45 micron membrane placed on top of TAP agar and bombarded without a drying phase). Plates are incubated in low light for two to three weeks before colonies are counted. Fosmidomycin-resistant colonies are green (vs yellowish for susceptible cells) and transformants are characterized using skills as known in the art. This demonstrates use of orfs encoding the entire mevalonate pathway as a selectable marker for green algae and by virtue of its functioning demonstrates its utility for overproduction of isoprenoid metabolites in microalgae.

Example 20: Metabolic engineering in transplastomic grain crops (rice)

In another exemplified embodiment, an operon comprising orfs encoding the entire mevalonate pathway are inserted into the plastids of rice as follows: A DNA fragment isolated from pHKO3, containing the complete mevalonate pathway, or from pFHO2, containing orfs encoding the entire mevalonate pathway and IPP isomerase, is inserted into the NcoI-XbaI sites of plasmid pMSK49 to replace the gfp coding region adjacent to the coding region for streptomycin resistance, aadA; or inserted into the BstXI-NcoI digested DNA of plasmid pMSK48 using skills as is known in the art for direct selection on fosmidomycin. The resulting plasmids contain rice-specific insertion sequences of pMSK35 as described in Khan and Maliga, Nature Biotechnology 17: 910-914, 1999. Embryonic suspensions, induced as previously described (Khan and Maliga 1999), of japonica rice *Oryza sativa* 'Taipei 309' engineered with the beta-carotene pathway (Ye *et al.* Science 287:303-305) are plated into filter paper and bombarded with the PDS1000 He device as described in Example 17. After two days on non-selective medium and then one to two weeks in selective AA medium (Toriyama and Hinata, Plant Science 41: 179-183, 1985) tissue is transferred to agar solidified medium of MS salts, and vitamins, 100mg/L myo-inositol, 4 mg/L 6-benzylaminopurine, 0.5 mg/L

indoleacetic acid, 0.5 mg/L1-napthaleneacetic acide, 3% sucrose, 4% maltose and 100 mg/L streptomycin sulfate or 500 μM fosmidomycin. Transplastomic shoots appear following cultivation in the light after three weeks and leaf samples are analyzed for the operon by PCR.

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